PATENT APPLICATION

NOVEL CO-STIMULATORY MOLECULES

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Novel Co-Stimulatory Molecules

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a continuation-in-part application of U.S. Patent

Application Serial No. 09/888,324, filed June 22, 2001 and International Patent Application

Serial No. PCT/US01/19973, which claim priority to and benefit of U.S. Provisional Patent

10 Application Serial Nos. 60/213,946, filed on June 23, 2000, and 60/241,245, filed on

October 17, 2000, the disclosure of each of which is incorporated herein by reference in its

entirety for all purposes.

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FIELD OF THE INVENTION

25 The present invention relates generally to polynucleotides and polypeptides

encoded therefrom, as wells as vectors, cells, antibodies, and methods for using and

producing the polynucleotides and polypeptides.

BACKGROUND OF THE INVENTION

T cells are a crucial component of the immune system. Not only is T cell

30 activation required for all specific immune responses against infectious agents, but T cells

also play an important role in tumor immunity and in autoimmune and allergic diseases. T

cell activation is initiated when T cells recognize their specific antigen (Ag) in the context

of major histocompatibility complex (MHC) molecules. T cell activation is well known by those of ordinary skill in the art and is characterized by such things as, e.g., cytokine synthesis, induction of various activation markers such as CD25 (interleukin-2 (IL-2) receptor), etc. CD4+ T cells recognize their immunogenic peptides in the context of MHC

5   class II molecules, whereas CD8+ T cells recognize their immunogenic peptides in the context of MHC class I molecules. For induction of T cell activation, cytokine synthesis or effector function, a second signal, mediated through CD28, is required. Two ligands for CD28 are B7-1 (CD80) and B7-2 (CD86). B7-1 and B7-2 are termed co-stimulatory molecules and are typically expressed on professional antigen-presenting cells (APCs). In

10   addition to binding the CD28 receptor, B7-1 and B7-2 also bind the CTLA-4 (CD152) receptor on T cells.

B7 molecules mediate both positive and negative signals to T cells by binding to CD28 and CTLA-4 (CD152) molecules on T cells. CTLA-4 is a negative regulator of the immune system. In general, wild-type (WT) B7-1, e.g., human B7-1,

15   preferentially binds CTLA-4 more strongly than it binds CD28. Typically, wild-type B7-1, e.g., human B7-1, binds CTLA-4 with about 100 times greater affinity than it binds CD28. Binding of B7-1 or B7-2 to CTLA-4 suppresses activation of T cells, resulting in reduced T cell proliferation and cytokine production (see, e.g., Walunas, T. L. et al. (1994) Immunity 1(5):405-413; Alegre, M. L. et al. (1998) J Immunol 161(7):3347-3356). Interaction

20   between B7-1 or B7-2 and CTLA-4 expressed on T cells down-regulates T cell responses and raises thresholds required for activation by CD28. Blockade of CTLA-4/ligand interactions can also augment in vivo tumor immunity (Leach, D. et al. (1996) Science 271:1734-1736). Consequently, CD28 and CTLA-4 play a pivotal role in the regulation of T cell activation and both are essential for proper functioning of the immune system. For

25   example, CD28 deficient mice are severely immunodeficient and show poor antigen specific T cell responses, while CTLA-4 deficient mice die of lymphoproliferative disease, show T cell expansion mediated by CD28 signaling and have a lack of down-regulation of T cell receptor signaling. Upon ligation by the co-stimulatory molecules B7-1 or B7-2, CD28 mediates a co-stimulatory signal that synergizes with T cell receptor signaling to induce,

30   e.g., proliferation, cytokine production and effector functions by both CD4+ and CD8+ T cells (proliferation/activation). Ligation of CTLA-4 with B7-1 (CD80) or B7-2 (CD86), however, dampens the CD80 or CD86 activating signal through CD28, resulting in down-

regulation of T cell activation. CD28 ligation reduces the inhibition mediated through the CTLA-4 signaling. CTLA-4 ligation mediates tolerance and anergy.

CD28 and CTLA-4 are both involved in the generation of an immune response to genetic vaccinations (e.g., nucleic acid vaccinations (NAV), DNA vaccinations, and viral vectors). CD28 deficient mice are unable to mount T cell or antibody responses against Beta-galactosidase (Beta-gal) when immunized with a plasmid encoding the Beta-gal gene, and CTLA-4 ligation suppresses the antibody response to Beta-gal in immunized wild-type mice (Horspool, J. et al. (1998), J Immunol 160:2706-2714). Expression of B7-1 on human myeloma cells (Wendtner, C. et al. (1997) Gene Therapy 4(7):726-735), murine mammary tumors (Martin-Fontecha, A. et al.(2000) J Immunol 164(2):698-704) or murine sarcoma (Indrova et al. (1998) Intl J Onc 12(2):387-390) enhances anti-tumor immunity. Furthermore, transfection of human APCs with retroviral vectors encoding B7-1 and tumor antigens induces a stronger cytotoxic T-lymphocyte (CTL) response than transfection with similar vectors encoding the tumor antigens alone (Zajac, P. et al. (1998) Cancer Res 58(20):4567-4571). Anti-viral responses are also modulated by co-stimulatory molecules. For example, DNA vaccination of chimpanzees and mice with HIV antigens in conjunction with B7-2 augmented anti-viral responses (Kim, J. et al. (1998) Vaccine 16(19):1828-1835; Tsuji et al. (1997) Eur J Immunol 27(3):782-787).

The binding properties of B7-1 and B7-2 have limited their usefulness in clinical applications. The present invention addresses needs for molecules having varied abilities to preferentially bind to and/or signal through either CD28 or CTLA-4 receptor and methods of using such molecules for selected and differential manipulation of T cell responses in vitro, ex vivo, and in vivo methods. Such molecules would be of beneficial use in a variety of applications, including, e.g., therapeutic and prophylactic treatments and vaccinations. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides novel co-stimulatory molecules (abbreviated as "NCSM") molecules, including polypeptides and proteins, related fusion polypeptide or fusion protein molecules, or functional equivalents thereof, homologues, and fragments of said polypeptide and protein molecules or equivalents, analogs, or derivatives thereof, and vectors, cells, and compositions comprising such NCSM molecules. The invention also provides nucleic acids encoding any of these polypeptides, proteins, fragments or variants thereof. In addition, the invention provides vectors, cells, and

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compositions comprising such nucleic acids, and uses of such NCSM polypeptides and NCSM nucleic acids; and other features are apparent upon further review.

Generally speaking, a "co-stimulatory molecule" refers to a molecule that acts in association or conjunction with, or is involved with, a second molecule or with
5 respect to an immune response in a co-stimulatory pathway. In one aspect, a co-stimulatory molecule may be an immunomodulatory molecule that acts in association or conjunction with, or is involved with, another molecule to stimulate or enhance an immune response. In another aspect, a co-stimulatory molecule is immunomodulatory molecule that acts in association or conjunction with, or is involved with, another molecule to inhibit or suppress
10 an immune response. A "co-stimulatory molecule" need not act simultaneously with or by the same mechanism as the second molecule. The term "NCSM" in reference to a molecule is not intended to limit the molecule to only those molecules that have positive co-stimulatory properties (e.g., that stimulate or augment T cell proliferation). In the initial recombination procedures described below, libraries of recombinant molecules were
15 generated by recombining nucleotide sequences of parental co-stimulatory molecules (CSM) as discussed herein. As shown by the data and analyses presented herein, novel recombinant molecules having a variety of properties were identified and selected. For example, polypeptide and nucleic acid molecules that enhance an immune response, such as by inducing T cell activation or proliferation (e.g., agonists), and molecules that down-
20 regulate or inhibit an immune response, such as by inhibiting T cell activation or proliferation (e.g., antagonists) were identified and selected. Further, molecules that preferentially bind and/or signal through either or both the CD28 and CTLA-4 receptors were identified and selected. Thus, the term "NCSM" refers to a co-stimulatory molecule and is not limited to molecules having the co-stimulatory properties of the parent sequences,
25 but is intended to refer collectively to all polypeptides of the invention, and nucleic acids encoding them, and other embodiments as described herein, unless specifically noted otherwise.

The term "NCSM" also includes variants, mutants, derivatives, and fragments of: 1) B7-1 and B7-2 polypeptides and nucleic acids, and 2) B7-1 and B7-2
30 polypeptides and nucleic acids of the Artiodactyla family (including, e.g., bovine B7-1 and B7-2), including all such polypeptide variants (and nucleic acids encoding such polypeptide variants) that exhibit properties similar or equivalent to the properties of the CD28BPs or CTLA-4BPs described herein. For example, the term includes B7-1, B7-2, and Artiodactyla

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(e.g., bovine) B7-1 and B7-2 polypeptide variants (and nucleic acids encoding such polypeptide variants) of the invention, wherein such polypeptide variants have a CD28/CTLA-4 binding affinity ratio about equal to, equal to, or greater than the CD28/CTLA-4 binding affinity ratio of hB7-1 or hB7-2 and/or an ability to induce a T-cell

5   proliferation, and/or a T-cell activation response about equal to, equal to, or greater than that of hB7-1. The term "NCSM" also is intended to include B7-1, B7-2, and Artiodactyla (e.g., bovine) B7-1 and B7-2 polypeptide variants (and nucleic acids encoding such polypeptide variants), wherein such polypeptide variants have a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of hB7-1 or hB7-2,

10   and/or an ability to induce a T-cell proliferation and/or T-cell activation response about equal to or less than that of hB7-1 or hB7-2.

In one aspect, the invention includes isolated or recombinant NCSM polypeptides, variants, homologues, derivatives, analogs, and fragments thereof. The invention includes recombinant NCSM polypeptides having varied abilities to preferentially

15   bind to and/or signal through CD28 and/or CTLA-4 receptor and provide for selected and differential manipulation of T cell responses in vitro, ex vivo, and in vivo. The invention also includes isolated or recombinant NCSM nucleic acids, variants, homologues, derivatives, analogs, and fragments thereof that encode polypeptides having varied abilities and uses described above. Such NCSM polypeptide and polynucleotides are useful in a

20   variety of applications, including e.g., therapeutic and prophylactic treatment methods, vaccinations, and diagnostic assays described below. The invention also provides NCSM polypeptides, and polynucleotide encoding such polypeptides, that strongly or preferentially bind at least one of CD28 or CTLA-4, but do not effectuate signaling; such molecules are useful in methods as potential antagonists of endogenous molecules, such as e.g.,

25   endogenous co-stimulatory molecules. Further, the invention provides NCSM polypeptides, and polynucleotides encoding them, having improved or altered receptor/ligand binding affinities and methods of using such molecules, including in pharmaceutical, prophylactic, therapeutic, vaccine, and diagnostic applications.

In one aspect, the invention provides an isolated or recombinant polypeptide

30   comprising an amino acid sequence of an , said extracellular domain (ECD) amino acid sequence having at least about 75% amino acid sequence identity to an extracellular domain amino acid sequence of, or the full-length sequence of, at least one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, and is not a naturally-occurring extracellular domain amino

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acid sequence, and wherein said polypeptide has a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1 and/or has an ability to induce a T-cell proliferation and/or T-cell activation response about equal to or greater than that of hB7-1. Some such polypeptides induce T-cell proliferation

5   or T-cell activation or both T-cell proliferation and T-cell activation. In some embodiments, the T cell activation or proliferation response is at least about equal to or greater than that cause by WT hB7-1.

Some such polypeptides of the invention may comprise an amino acid sequence having 75% identity to a full-length polypeptide sequence of SEQ ID NOS:48-68,

10   174-221, 283-285, and 290-293. Such polypeptides may be expressed on the surface of a cell membrane (e.g., following transfection of the cell with a nucleic acid that encodes said polypeptide) or associated with or bound to a cell membrane, or form an integral membrane protein (e.g., by further comprising a transmembrane domain amino acid sequence). Through such expression, such polypeptides typically become integral membrane proteins.

15   Preferably, such cell-expressed polypeptides, membrane-associated, or membrane-bound polypeptides, have an ability to induce a T cell proliferation response that is equal to or greater than that induced by hB7-1.

Other such polypeptides modulate T-cell activation, but do not induce proliferation of purified T-cells activated by soluble anti-CD3 mAbs.

20   In one embodiment, the isolated or recombinant polypeptide comprises an ECD that has at least about 90% amino acid sequence identity to an ECD or the full-length sequence of at least one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293. In another embodiment, the ECD amino acid sequence comprises an amino acid subsequence of at least one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293.

25   Some such isolated or recombinant polypeptides are soluble (e.g., not cell membrane-bound or membrane-associated or forming an integral part of a cell membrane). The invention includes monomeric and multimeric (or aggregated) forms of such soluble polypeptides. A soluble monomer comprises one soluble polypeptide of the invention (e.g., one soluble NCSM-ECD), while a soluble multimer or soluble aggregate comprises at least

30   two soluble polypeptides of the invention (e.g., two soluble ECDs). The multimer or aggregate may be formed by cross-linking or other means to link polypeptide sequences. Such polypeptides may comprise a fusion protein comprising at least one additional amino acid sequence, such as an Ig polypeptide. Some such soluble polypeptides, in the presence

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of a population of activated T cells, have an ability to induce a T cell proliferation response, in the presence of a population of activated T cells, that is less than the T cell proliferation response induced by a soluble human B7-1 polypeptide in the presence of a population of activated T cells.

Some such isolated or recombinant polypeptides further comprise at least one of a signal peptide domain, transmembrane domain (TMD), and/or cytoplasmic domain(CD), including, e.g., wherein such domain is a WT, variant, or mutant domain of a co-stimulatory polypeptide, including, e.g., a recombinant domain derived from a mammalian B7-1 or B7-2. In one embodiment, the isolated or recombinant polypeptide comprises an amino acid subsequence, including any of a signal peptide, TMD, or CD of any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293. Nucleic acids encoding such polypeptides and domains are also provided.

In another aspect, the invention provides an isolated or recombinant polypeptide, which polypeptide comprises a non-naturally-occurring amino acid sequence encoded by a nucleic acid comprising a polynucleotide sequence selected from the group of: (a) a polynucleotide sequence selected from SEQ ID NOS:1-21 and 95-142, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which, but for the degeneracy of the genetic code, hybridizes under at least stringent or highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b); (d) a polynucleotide sequence comprising all or a nucleotide fragment of (a), (b), or (c), wherein the nucleotide fragment encodes a polypeptide having a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1, and/or has an ability to induce a T-cell proliferation and/or T-cell activation response about equal to or greater than that of hB7-1; (e) a polynucleotide sequence encoding a polypeptide, the polypeptide comprising an amino acid sequence which is substantially identical over at least about 150 contiguous amino acid residues of any one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293; and (f) a polynucleotide sequence encoding a polypeptide that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1, and/or has an ability to induce T-cell proliferation or activation or both that is about equal to or greater than that induced by

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hB7-1, which polynucleotide sequence has at least about 70% amino acid sequence identity to at least one polynucleotide sequence of (a), (b), (c), or (d).

Also provided is an isolated or recombinant polypeptide comprising an amino acid sequence according to the formula: MGHTM-X6-W-X8-SLPPK-X14-PCL-X18-X19-X20-QLLVLT-X27-LFYFCSGITPKSVTKRVKETVMLSCDY-X55-TSTE-X60-LTSLRIYW-X69-KDSKMVLAILPGKVQVWPEYKNRTITDMNDN-X101-RIVI-X106-ALR-X110-SD-X113-GTYTCV-X120-QKP-X124-LKGAYKLEHL-X135-SVRLMIRADFPVP-X149-X150-X151-DLGNPSPNIRRLICS-X167-X168-X169-GFPRPHL-X177-WLENGEELNATNTT-X192-SQDP-X197-T-X199-LYMISSEL-X208-FNVTNN-X215-SI-X218-CLIKYGEL-X227-VSQIFPWSKPKQEPPIDQLPF-X249-VIIPVSGALVL-X261-A-X263-VLY-X267-X268-ACRH-X273-ARWKRTRRNEETVGTE RLSPIYLGSAQSSG (SEQ ID NO:284), or a subsequence thereof comprising an extracellular domain, wherein position X6 is Lys or Glu; position X8 is Arg or Gly; position X14 is Arg or Cys; position X18 is Trp or Arg; position X19 is Pro or Leu; position X20 is Ser or Pro; position X27 is Asp or Gly; position X55 is Asn or Ser; position X60 is Glu or Lys; position X69 is Gln or Arg; position X101 is Pro or Leu; position X106 is Leu or Gln; position X110 is Pro or Leu; position X113 is Lys or Ser; position X120 is Val or Ile; position X124 is Val or Asp; position X135 is Thr or Ala; position X149 is Thr, Ser, or del; position X150 is Ile or del; position X151 is Asn or Thr; position X167 is Thr or del; position X169 is Ser or del; position X169 is Gly or del; position X177 is Cys or Tyr; position X192 is Val or Leu; position X197 is Gly or Glu; position X199 is Glu or Lys; position X208 is Gly or Asp; position X215 is His or Arg; position X218 is Ala or Val; position X227 is Ser or Leu; position X249 is Trp, Leu, or Arg; position X261 is Ala or Thr; position X263 is Val, Ala, or Ile; position X267 is Arg or Cys; position X268 is Pro or Leu; and position X273 is Gly or Val. Typically, the ECD comprises at least about amino acids 35 to 244 or amino acids 35 to 255 of SEQ ID NO:284. Some such polypeptides have a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1, and/or has an ability to induce T-cell proliferation or activation or both that is about equal to or greater than that induced by hB7-1.

In yet another aspect, the invention provides an isolated or recombinant polypeptide comprising a subsequence of an amino acid sequence set forth in any of SEQ

ID NOS:48-68, 174-182, 184-221, 283-285, and 290-293, wherein the subsequence is the extracellular domain of said amino acid sequence.

A soluble form of an NCSM polypeptide or a human B7-1 polypeptide typically comprises a polypeptide, comprising at least one ECD, which is not expressed on the surface of a cell, is not embedded in a cell membrane, and/or is not associated with a lipid bilayer. Such soluble polypeptides may contain a TD or a fragment thereof such that the polypeptide remains soluble (e.g., is not embedded membrane as an integral membrane protein or associated with a lipid bilayer). Such soluble polypeptides may also include one or more additional amino acid sequences, such as an Fc portion of an Ig (e.g., IgG). Such soluble polypeptide may comprise an ECD monomer, ECD-Ig fusion protein monomer, ECD multimer or aggregate, or ECD-Ig fusion protein multimer or aggregate (see data and discussion below).

Also provided are isolated or recombinant polypeptides, each of which comprise an amino acid sequence of an extracellular domain, wherein said extracellular domain amino acid sequence has at least about 75% amino acid sequence identity to an extracellular domain amino acid sequence of at least one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, and is not a naturally-occurring extracellular domain amino acid sequence, wherein such polypeptide, in the presence of activated T cells, has an ability to down-regulate or inhibit a T cell proliferation response compared to the response caused by soluble hB7-1 (e.g., hB7-1-ECD or hB7-1-ECD-Ig) in the presence of activated T cells. The polypeptide can be either a soluble ECD monomer or a soluble ECD-Ig fusion protein. Such polypeptides may comprise a fusion protein comprising at least one additional polypeptide, such as, e.g., an Ig polypeptide.

The invention also includes each nucleic acid comprising a polynucleotide sequence (including degenerate sequences) that encodes each such isolated or recombinant polypeptide comprising a soluble polypeptide as described above, and a complementary polynucleotide sequence thereof. Polynucleotide sequences that, but for the degeneracy of the genetic code, hybridizes under at least stringent conditions over substantially the entire length of each such nucleic acid are also included.

The invention further provides isolated or recombinant polypeptides comprising an amino acid sequence having at least about 95% amino acid sequence identity to a full-length sequence of at least one of SEQ ID NOS:69-92, 222-252, 286-289, or to a subsequence thereof comprising the extracellular domain, wherein said sequence (a) is a

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non naturally-occurring sequence, and (b) comprises at least one of: Gly at position 2; Thr at position 4; Arg at position 5; Gly at position 8; Pro at position 12; Met at position 25; Cys at position 27; Pro at position 29; Leu at position 31; Arg at position 40; Leu at position 52; His at position 65; Ser at position 78; Asp at position 80; Tyr at position 87; Lys at position

5   120; Asp at position 122; Lys at position 129; Met at position 135; Phe at position 150; Ile at position 160; Ala at position 164; His at position 172; Phe at position 174; Leu at position 176; Asn at position 178; Asn at position 186; Glu at position 194; Gly at position 196; Thr at position 199; Ala at position 210; His at position 212; Arg at position 219; Pro at position 234; Asn at position 241; Leu at position 244; Thr at position 250; Ala at position 254; Tyr

10   at position 265; Arg at position 266; Glu at position 273; Lys at position 275; Ser at position 276; an amino acid deletion at position 276; or Thr at position 279, wherein the position number corresponds to that of the human B7-1 amino acid sequence (SEQ ID NO:278), wherein said polypeptide has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1, and/or an ability to

15   induce a T-cell proliferation or T-cell activation response about equal to or less than that of hB7-1. A subsequence comprises signal peptide, EDC, TMD, or CD. Each such polypeptide may further comprise at least one additional amino acid sequence, including, e.g., a sequence corresponding to a signal peptide, TMD, ECD, or CD.

In another aspect, the invention provides isolated or recombinant

20   polypeptides, each comprising an amino acid sequence that differs from the amino acid sequence of a primate B7-1, wherein the difference between the amino acid sequence of the polypeptide and the amino acid sequence of the primate B7-1 comprises a different amino acid at at least one amino acid residue position selected from the group consisting of 12, 25, 27, 29, 40, 52, 65, 122, 129, 135, 164, 174, 196, 199, 210, 219, 234, 241, 254, 275, 276, and

25   279, wherein the amino acid residue positions correspond to the amino acid residue positions in the amino acid sequence of human B7-1 of SEQ ID NO:278. For some such polypeptides, the different amino acid comprises at least one of: Pro at position 12; Met at position 25; Cys at position 27; Pro at position 29; Arg at position 40; Leu at position 52; His at position 65; Asp at position 122; Lys at position 129; Met at position 135; Ala at

30   position 164; Phe at position 174; Gly at position 196; Thr at position 199; Ala at position 210; Arg at position 219; Pro at position 234; Asn at position 241; Ala at position 254; Lys at position 275; Ser at position 276; or Thr at position 279. Preferably, for some such polypeptides, the different amino acid is His at position 65.

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In another aspect, the invention provides isolated or recombinant polypeptides comprising an amino acid sequence that differs from a primate B7-1 sequence in at least one mutation selected from: Ser 12 Pro; Leu 25 Met; Gly 27 Cys; Ser 29 Pro; Lys 40 Arg; His 52 Leu; Tyr 65 His; Glu 122 Asp; Glu 129 Lys; Thr 135 Met; Thr 164 Ala;

5   Ser 174 Phe; Glu 196 Gly; Ala 199 Thr; Thr 210 Ala; Lys 219 Arg; Thr 234 Pro; Asp 241 Asn; Val 254 Ala; Arg 275 Lys; Arg 276 Ser; or Arg 279 Thr; the mutation being indicated relative to human B7-1 with the amino acid sequence shown in SEQ ID NO:278, wherein said sequence does not occur in nature, and wherein said polypeptide has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio

10   of human B7-1, and/or an ability to induce a T-cell proliferation and/or T-cell activation response about equal to or less than that of hB7-1.

Also included are isolated or recombinant polypeptides comprising an amino acid sequence having at least about 75% sequence identity to at least one of SEQ ID NOS:263-272, or a subsequence thereof comprising the extracellular domain, where the

15   amino acid sequence is not naturally-occurring, and the polypeptide has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1, and/or an ability to induce a T-cell proliferation and/or activation response about equal to or less than that of hB7-1.

In yet another aspect, the invention includes an isolated or recombinant

20   polypeptides which comprises a non naturally-occurring amino acid sequence encoded by a nucleic acid comprising a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:22-45, 143-173, 253-262, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:69-92, 222-247, 263-272, 286-289, or a complementary

25   polynucleotide sequence thereof; (c) a polynucleotide sequence which, but for the degeneracy of the genetic code, hybridizes under highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b); (d) a polynucleotide sequence comprising all or a fragment of (a), (b), or (c), wherein the fragment encodes a polypeptide having a CTLA-4/CD28 binding affinity ratio about equal to or greater than the

30   CTLA-4/CD28 binding affinity ratio of human B7-1, or an ability to induce a T-cell proliferation or activation response about equal to or less than that of hB7-1; (e) a polynucleotide sequence encoding a polypeptide, the polypeptide comprising an amino acid sequence which is substantially identical over at least about 150 contiguous amino acid

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residues of any one of SEQ ID NOS:69-92, 222-247, 263-272, 286-289, and (f) a polynucleotide sequence encoding a polypeptide that has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1 or an ability to induce a T-cell proliferation or activation response about equal to or less

5   than that of hB7-1, which polynucleotide sequence has at least about 70% identity to at least one polynucleotide sequence of (a), (b), (c), or (d).

The invention also includes an isolated or recombinant polypeptide comprising an amino acid sequence according to the formula:

MGHTRRQGTSP-X12-KCPYLKFFQLLV-X25-ACL-X29-
10   HLCSGVIHVT-X40-EVKEVATLSCGLNVSVEELAQTRIHWQKEKKMVLTM
MSGDMNIWPEYKNRTIFDITNNLSIVILALRPSDEGTYECVVLKY-X122-KDAFKR-
X129-HLAEVMLSVKAD FPTPSITDFEIPPSNIRRIICS-X164-
SGGFPEPHLFWLENGEELNAINTTVSQDPET-X196-LYTVSSKLDFNM
TANHSFMCLI-X219-YGHLRVNQTFNWNTPKQEHFP-X241-NLLPSWA
15   ITLISANGIFVICCLTYRFAPRCRERKSNETLRRESVCPV (SEQ ID NO:287), or a subsequence thereof comprising the extracellular domain, wherein position X12 is Ser or Pro; position X25 is Leu or Met; position X29 is Ser or Pro; position X40 is Lys or Arg; position X122 is Glu or Asp; position X129 is Glu or Lys; position X164 is Thr or Ala; position X196 is Glu or Gly; position X219 is Lys or Arg; position X241 is Asp or Asn.
20   Some such polypeptides have a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of hB7-1, and/or ability to induce T-cell proliferation or activation or both about equal to or less than that induced by hB7-1.

The invention also provides an isolated or recombinant polypeptide comprising a subsequence of an amino acid sequence set forth in any of SEQ ID NOS:69-
25   92, 222-247, 263-272, and 286-289, wherein the subsequence is the extracellular domain or full-length sequence of such amino acid sequence. Furthermore, the invention includes the full-length polypeptide sequence and one or more subsequences thereof, e.g., signal peptide, extracellular domain (ECD), transmembrane domain (TMD), and/or cytoplasmic domain (CD) of any of SEQ ID NOS:66, 81, 85, 86, 88, 90, 91, 285, 288, 289, 291, and 294, and
30   nucleic acid sequences encoding any of these amino acid sequences.

The invention provides isolated or recombinant nucleic acids comprising a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:1-21 and 95-142, or a complementary polynucleotide sequence thereof; (b) a

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polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which, but for codon degeneracy, hybridizes under at least stringent or highly stringent conditions over substantially the entire length of polynucleotide

5   sequence (a) or (b); and (d) a polynucleotide sequence comprising all or a nucleotide fragment of (a), (b), or (c), wherein the fragment encodes a polypeptide having a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1, or an ability to induce a T-cell proliferation or activation response about equal to or greater than that of hB7-1. In some such nucleic acids,

10   the polynucleotide sequence of (d) encodes a nucleotide fragment of (a) or (b) that encodes an ECD having a CD28/CTLA-4 binding affinity ratio or an ability to induce a T-cell proliferation or activation response about equal to or greater than that of hB7-1.

       The invention also includes isolated or recombinant nucleic acids comprising a polynucleotide sequence encoding a polypeptide, wherein the encoded polypeptide

15   comprises an amino acid sequence which is (a) substantially identical over at least about 150 or 200 contiguous amino acid residues of any one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293 and (b) is a non naturally-occurring sequence.

       In addition, the invention includes isolated or recombinant nucleic acids comprising a nucleotide sequence coding for a polypeptide comprising the amino acid

20   sequence set forth in any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a subsequence thereof, wherein the subsequence comprises at least one of: the signal sequence, extracellular domain, or transmembrane domain of said polypeptide, and the cytoplasmic domain of said polypeptide, and wherein the amino acid sequence or subsequence is a non naturally-occurring sequence. Similarly, fragments of the above

25   nucleotides that encode a polypeptide that has a substantially equivalent or equivalent binding activity of a NCSM polypeptide molecule, produces a substantially equivalent or equivalent NCSM-polypeptide-mediated immune response, e.g., induction or inhibition of T cell activation or proliferation, or cytokine production are a feature.

       Also provided is an isolated or recombinant nucleic acid comprising a

30   polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:22-45, 143-173, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:69-92, 222-247, 286-289, or a complementary polynucleotide sequence thereof; (c) a polynucleotide

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sequence which, but for the degeneracy of the genetic code, hybridizes under highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b); and (d) a polynucleotide sequence comprising all or a fragment of (a), (b), or (c); wherein (c) or (d) encodes a polypeptide having a non naturally-occurring sequence

5   comprising at least one of:

Gly at position 2; Thr at position 4; Arg at position 5; Gly at position 8; Pro at position 12; Met at position 25; Cys at position 27; Pro at position 29; Leu at position 31; Arg at position 40; Leu at position 52; His at position 65; Ser at position 78; Asp at position 80; Tyr at position 87; Lys at position 120; Asp at position 122; Lys at position 129; Met at

10   position 135; Phe at position 150; Ile at position 160; Ala at position 164; His at position 172; Phe at position 174; Leu at position 176; Asn at position 178; Asn at position 186; Glu at position 194; Gly at position 196; Thr at position 199; Ala at position 210; His at position 212; Arg at position 219; Pro at position 234; Asn at position 241; Leu at position 244; Thr at position 250; Ala at position 254; Tyr at position 265; Arg at position 266; Glu at

15   position 273; Lys at position 275; Ser at position 276; an amino acid deletion at position 276; and Thr at position 279, wherein the position number corresponds to that of the human B7-1 amino acid sequence (SEQ ID NO:278), and wherein said polypeptide has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1.

20   The invention further provides an isolated or recombinant nucleic acid comprising a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:253-262, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:263-272, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which,

25   but for codon degeneracy, hybridizes under highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b) and encodes a polypeptide having a non naturally-occurring sequence; and (d) a polynucleotide sequence comprising all or a fragment of (a), (b), or (c), wherein the fragment encodes a polypeptide having (i) a non naturally-occurring sequence and (ii) a CTLA-4/CD28 binding affinity ratio about equal to

30   or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1, or an ability to induce a T-cell proliferation or activation response about equal to or less than that of hB7-1.

The invention also includes an isolated or recombinant nucleic acid comprising a polynucleotide sequence encoding a polypeptide that comprises an amino acid

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sequence which is substantially identical over at least about 150 contiguous amino acid residues of any one of SEQ ID NOS:69-92, 222-247, 263-272, and 286-289.

The invention also provides an isolated or recombinant nucleic acid comprising a nucleotide sequence coding for a polypeptide comprising the amino acid sequence set forth in any of SEQ ID NOS:69-92, 222-247, 263-272, and 286-289, or a subsequence thereof, wherein the subsequence comprises at least one of the signal sequence, ECD, transmembrane domain, and cytoplasmic domain of said polypeptide, and the amino acid sequence or subsequence is a non naturally-occurring sequence.

In another aspect, the invention provides an isolated or recombinant nucleic acid encoding a polypeptide that has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1, or an ability to induce a T-cell proliferation and/or activation response about equal to or greater than that of hB7-1, produced by mutating or recombining at least one nucleic acids described above. Also included is an isolated or recombinant polypeptide comprising an amino acid sequence having the formula:

MGHTMKWGSLPPKRPCLWLSQLLVLTGLFYFCSGITPK-
SVTKRVKETVM-X50-SCDY-X55-X56-STEELTSLRIYWQKDSKMVL
AILPGKVQVWPEYKNRTITD-MNDNPRIVILALRLSD-X113-GTYTCV-X120-QK-
X123-X124-X125-X126-G-X128-X129-X130-X131-EHL-X135-SV-X138-L-X140-
IRADFPVPSITDIGHPAPNVK RIRCSASG-X170-FPEPRLAWMEDGEEL-NAVNTTV-
X193-X194-X195-LDTELYSVSSELD-X209-N-X211-
TNNHSIVCLIKYGELSVSQIFPWSKPK QEPPIDQLPFWVI-X252-X253-
VSGALVLTAVVLYCLACRHVAR (SEQ ID NO:290), or a subsequence thereof
comprising the extracellular domain, wherein position X50 is Leu or Pro; position X55 is
Asn or Ser; position X56 is Ala or Thr; position X113 is Ser or Lys; position X120 is Ile or
Val; position X123 is Pro or deleted; position X124 is Val, Asn, or Asp; position X125 is
Leu or Glu; position X126 is Lys or Asn; position X128 is Ala or Ser; position X129 is Tyr
or Phe; position X130 is Lys or Arg; position X131 is Leu or Arg; position X135 is Ala or
Thr; position X138 is Arg or Thr; position X140 is Met or Ser; position X170 is Asp or Gly;
position X193 is Asp or is deleted; position X194 is Gln or is deleted; position X195 is Asp
or is deleted; position X211 is Val or Ala; position X252 is Ile or Val; and position X253 is
Leu or Pro. The polypeptide may comprise a sequence of any of SEQ ID NOS:59, 62, 180,
184, 188, 195, 196, 200, 201, 204, 211, 213, 219, and 291. Some such polypeptides have a

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CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1, and/or an ability to induce T-cell proliferation and/or activation about equal to or greater than that induced by hB7-1.

Another feature of the invention is an isolated or recombinant polypeptide
5   comprising an amino acid sequence according to the formula:

MGHTMKWG-X9-LPPKRPCLWLSQLLVLTGLFYFCSG-X35-
TPKSVTKRV KETVMLSCDY-X55-TSTEELTSLRIYWQKDSKMVLAILPGKVQVW
PEYKNRTITDMNDNPRIVILALR-X110-SDSGTYTCVIQKP-X124-LKGAYKLEHL-
X135-SVRLMIRADFPVPTINDLGNPSPNIRRLICSTSGGFPRPHLYWLENG-X183-
10   ELNATNTT-X192-SQDPETKLYMISSELDFN-X211-TSN-X215-X216-X217-
LCLVKYGDLTVSQ-X231-FYWQESKPTPSANQHLTWTIIIPVSAFGISVIIAVI
LTCLTCRNAAIRRQRRENEV-X288-M-X290-SCSQSP (SEQ ID NO:292), or a
subsequence thereof comprising the extracellular domain, wherein position X9 is Thr or
Ser; position X35 is Ile or Thr; position X55 is Asn or Ser; position X110 is Leu or Pro;
15   position X124 is Asp or Val; position X135 is Thr or Ala; position X183 is Lys or Glu;
position X192 is Leu or Val; position X211 is Met or Thr; position X215 is His or is
deleted; position X216 is Ser or is deleted; position X217 is Phe or is deleted; position X231
is Thr or Ser; position X288 is Lys or Glu; position X290 is Glu or Gln, and wherein said
amino acid sequence is a non naturally-occurring sequence. Some such polypeptides have a
20   CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1, and/or an
ability to induce T-cell proliferation and/or activation about equal to or greater than that
induced by hB7-1.

The invention includes an isolated or recombinant polypeptide comprising
the amino acid sequence of SEQ ID NO:93 or SEQ ID NO:94, or a subsequence thereof,
25   wherein the subsequence comprises at least one of the signal sequence, ECD,
transmembrane domain, and cytoplasmic domain of said polypeptide. Also provided is an
isolated or recombinant nucleic acid comprising a polynucleotide sequence selected from:
(a) a polynucleotide sequence selected from SEQ ID NO:46 or SEQ ID NO:47, or a
complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a
30   polypeptide selected from SEQ ID NO:93, SEQ ID NO:94, or a complementary
polynucleotide sequence thereof; (c) a polynucleotide sequence encoding a subsequence of
a polypeptide selected from SEQ ID NO:93, SEQ ID NO:94, or a complementary
polynucleotide sequence thereof, wherein the subsequence comprises at least one of: the

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signal sequence, extracellular domain, transmembrane domain, and cytoplasmic domain of the polypeptide.

In another aspect, the invention provides a polypeptide which is specifically bound by a polyclonal antisera raised against at least one antigen, the antigen comprising

5   the polypeptide sequence selected from any of SEQ ID NOS:48-94, 174-252, 263-272, 283-293, or a fragment thereof, wherein the antisera is subtracted with one or more (and optionally all) polypeptides encoded by one or more of the sequences set forth at GenBank Nucleotide Accession Nos: A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896,

10   AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958,

15   Y08823, and Y09950. Some such polypeptides have a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1, and/or an ability to induce a T-cell proliferation or T-cell activation response about equal to or greater than that of hB7-1.

The invention further includes an antibody or antisera produced by

20   administering any NCSM polypeptide described above to a mammal, which antibody specifically binds at least one antigen, the antigen comprising a polypeptide comprising at least one amino acid sequence of any of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, which antibody does not specifically bind to a polypeptide encoded by at least one (optionally all) of the sequences at GenBank Nucleotide Accession Nos: A92749, A92750,

25   AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191,

30   S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950.

The invention provides an antibody or antisera which specifically binds a polypeptide which comprises any sequence selected from any of SEQ ID NOS:48-94, 174-

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252, 263-272, and 283-293, wherein the antibody or antisera does not specifically bind to at least one (optionally all) polypeptide encoded by at least one of GenBank Nucleotide Accession Nos: A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519,

5   AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823,

10   and Y09950. The antibodies are, e.g., polyclonal, monoclonal, chimeric, humanized, single chain, Fab fragments, fragments produced by an Fab expression library, or the like.

In another aspect, the invention provides a nucleic acid which comprises a unique subsequence in a nucleic acid selected from SEQ ID NOS:1-47, 95-173, and 253-262, wherein the unique subsequence is unique as compared to at least one (optionally all)

15   nucleic acid corresponding to any of GenBank Nucleotide Accession Nos.: A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904,

20   AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950. The invention also includes a polypeptide which comprises a unique subsequence in a polypeptide selected from: SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, wherein

25   the unique subsequence is unique as compared to at least one (optionally all) polypeptide encoded by any of GenBank Nucleotide Accession Nos. shown above.

The invention includes a target nucleic acid which, but for nucleotide codon degeneracy, hybridizes under stringent conditions to a unique coding oligonucleotide that encodes a unique subsequence in a polypeptide selected from SEQ ID NOS:48-94, 174-252,

30   263-272, and 283-293, wherein the unique subsequence is unique as compared to at least one (optionally all) polypeptide encoded by any of GenBank Nucleot. Access. Nos.: A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825,

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AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950.

The invention also includes compositions comprising any polypeptide and/or polynucleotide described herein in an excipient, preferably a pharmaceutically acceptable excipient. In one aspect, the invention provides compositions comprising an isolated or recombinant NCSM polypeptide comprising the amino acid sequence SEQ ID NOS:48-68, 174-221, 283-285, 290-293, or a costimulatory fragment thereof, wherein said costimulatory fragment has a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1, or an ability to induce a T-cell proliferation or activation response about equal to or greater than that of hB7-1, and a carrier or excipient. Compositions comprising an isolated or recombinant NCSM polypeptide comprising the amino acid sequence of SEQ ID NOS:69-92, 222-247, 263-272, 286-289, or a costimulatory fragment thereof, wherein said costimulatory fragment has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1, or an ability to induce a T-cell proliferation or activation response about equal to or less than that of hB7-1, and a carrier are also a feature of the invention.

Also included are isolated or recombinant polypeptides, each comprising an amino acid sequence corresponding to an extracellular domain, wherein said amino acid sequence has at least about 92%, 93%, 94%, 95% or more amino acid sequence identity to the amino acid sequence corresponding to the extracellular domain of SEQ ID NO:66, and wherein said polypeptide has a CD28/CTLA-4 binding affinity ratio greater than the CD28/CTLA-4 binding affinity ratio of human B7-1. Some such polypeptides may further comprise at least one further amino acid sequence corresponding to a signal peptide, transmembrane domain or a cytoplasmic domain.

The invention also includes isolated or recombinant polypeptide variants, each of which comprises an amino acid sequence that differs from the amino acid sequence of a primate B7-1, wherein the difference between the amino acid sequence of the variant and the amino acid sequence of the primate B7-1 comprises a different amino acid at position 65 other than alanine, wherein the position corresponds to the position in the amino

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acid sequence of human B7-1 of SEQ ID NO:278. The different amino acid may comprise His, Arg, Lys, Pro, Phe, or Trp, and the primate B7-1 may be hB7-1. Some such polypeptide variants have a CTLA-4/CD28 binding affinity ratio greater than that or hB7-1 or an ability to induce a T cell proliferation response less than that of hB7-1.

5   The invention also includes an isolated or recombinant nucleic acid comprising a polynucleotide sequence encoding a polypeptide, where the polypeptide comprises an amino acid sequence which is substantially identical over at least 175 contiguous amino acids of any one of those NCSM polypeptide sequences listed. In various embodiments, the encoded polypeptide comprises at least about 100, 150, 170, 180, 190,

10   200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 220, 225, 230, 240, 250, 260, 265, 270, 275, or 285 or more contiguous amino acid residues or substantially identical variants of any one of the polypeptide sequences listed, or encoded by any nucleic acid listed. These polypeptides can exist separately or as components of one of more fusion proteins.

           The invention also includes a cell comprising any nucleic acid described

15   herein, or which expresses any polypeptide or nucleic acid noted herein. In one embodiment, the cell expresses a polypeptide encoded by the nucleic acids herein.

           The invention also includes a vector comprising any nucleic acid of the invention. The vector can comprise a plasmid, a cosmid, a phage, or a virus or a virus-like particle (VLP) (or virus fragment); the vector can be, e.g., an expression vector, a cloning

20   vector, a packaging vector, an integration vector, or the like. The invention also includes a cell transduced by the vector. The invention also includes compositions comprising any nucleic acid described herein, and an excipient, preferably a pharmaceutically acceptable excipient. Cells and transgenic animals that include any polypeptide or nucleic acid herein, e.g., produced by transduction of the vector, are also a feature of the invention.

25   The invention also includes compositions produced by digesting one or more of the nucleic acids described herein with a restriction endonuclease, an RNAse, or a DNAse; and, compositions produced by incubating one or more nucleic acids described herein in the presence of deoxyribonucleotide triphosphates and a nucleic acid polymerase, e.g., a thermostable polymerase.

30   The invention also includes compositions comprising two or more nucleic acids described herein. The composition may comprise a library of nucleic acids, where the library contains at least 5, 10, 20 or 50 or more nucleic acids.

In another aspect, the invention includes an isolated or recombinant polypeptide encoded by any nucleic acid described herein. In one embodiment, the polypeptide may comprise a sequence selected from any of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293. These sequences and fragments thereof can be present separately or

5   as components of larger proteins such as fusion proteins.

Any polypeptide described herein optionally can effect or alter an immune response, e.g., either induce or inhibit proliferation or activation of T cells. In other embodiments, any polypeptide described above can bind preferentially either CD28 or CTLA-4 or both CD28 and CTLA-4 as described herein. In other embodiments, any

10   polypeptide described herein optionally can enhance or limit cytokine production as described herein. Nucleotides encoding any such polypeptides having these properties are also a feature of the invention.

In one class of embodiments, any polypeptide described herein may further include a secretion signal or localization signal sequence, e.g., a signal sequence, an

15   organelle targeting sequence, a membrane localization sequence, and the like. Any polypeptide described herein may further include a sequence that facilitates purification, e.g., an epitope tag (such as, e.g., a FLAG epitope), a polyhistidine tag, a GST fusion, and the like. The polypeptide optionally includes a methionine at the N-terminus. Any polypeptide described herein optionally includes one or more modified amino acids, such as

20   a glycosylated amino acid, a PEG-ylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, a carboxylated amino acid, a phosphorylated amino acid, an acylated amino acid, or the like. Any polypeptide described herein further may be incorporated into a fusion protein, e.g., a fusion with an immunoglobulin (Ig) sequence.

25   Methods for producing the polypeptides of the invention are also included. One such method comprises introducing into a population of cells any NCSM nucleic acid described herein, which is operatively linked to a regulatory sequence effective to produce the encoded polypeptide, culturing the cells in a culture medium to produce the polypeptide, and isolating the polypeptide from the cells or from the culture medium. Another such

30   method comprises introducing into a population of cells a recombinant expression vector comprising any NCSM nucleic acid described herein; administering the expression vector into a mammal; and isolating the polypeptide from the mammal or from a byproduct of the mammal.

The invention also includes a method of treating an autoimmune or allergic disorder in a subject in need of such treatment by administering to the subject an effective amount of any NCSM polypeptide (or polynucleotide or expression vector encoding such polypeptide) described herein. In various embodiments, the autoimmune disorder may be

5   multiple sclerosis, rheumatoid arthritis, lupus erythematosus, type I diabetes, psoriasis and the like.

The invention also includes a method of enhancing or reducing an immune response in a subject, such as either by inducing or inhibiting T cell proliferation or activation, by administration of at least one NCSM polypeptide and/or NCSM

10   polynucleotide described herein to a population of cells. The population of cells to which the nucleic acid or polypeptide is administered can be in vivo, ex vivo, or in vitro (e.g., cultured cells). The invention includes a method of inducing, modifying, or inhibiting T-cell proliferation, the method comprising contacting a population of T cells with a polypeptide of the invention, thereby inducing, modifying, or inhibiting, respectively,

15   proliferation of the T cells (relative to the response generated by WT hB7-1). Polypeptides that induce such T cell proliferation include CD28BP polypeptides; polypeptides that inhibit T cell proliferation include the CTLA-4BP polypeptides and B7-1 and B7-2 polypeptide variants discussed herein.

The invention also includes, in a method of treating a disorder or medical

20   condition treatable by administration of NCSM polypeptides (or fragments thereof) or NCSM polynucleotides (or fragments thereof) to a subject, an improvement comprising administering to the subject an effective amount of a polypeptide and/or nucleic acid (or fragments thereof) described herein. The disorder, disease, or medical condition treatable by administration of NCSM polypeptides and/or nucleic acids (or fragments thereof,

25   including soluble NCSMs and fusion proteins and vectors encoding them) may be, but is not limited to, e.g., chronic disease, autoimmune disorder, multiple sclerosis, rheumatoid arthritis, lupus erythematosus, type I diabetes, psoriasis, AIDS or AIDS-related complexes, allogeneic or xenogeneic grafts or transplants, a variety of cancers, viral and/or bacterial infections, or the like.

30   Also included is a method of therapeutic or prophylactic treatment of a disease or disorder in a subject in need of such treatment, comprising administering to the subject any NCSM polypeptide described herein and an immunogen specific for said

disease or disorder, wherein the combined amount of polypeptide and immunogen is effective to prophylactically or therapeutically treat said disease or disorder.

In yet another aspect, the invention includes a method of enhancing, diminishing, modifying, or potentiating an immune response in a subject, comprising:

5   directly administering to the subject a polynucleotide comprising any NCSM nucleic acid sequence described herein, operably linked to a promoter sequence that controls the expression of said nucleic acid sequence, said polynucleotide being present in an amount sufficient that uptake of said polynucleotide into one or more cells of the subject occurs and sufficient expression of said nucleic acid sequence results to produce an amount of a

10   polypeptide effective to enhance, diminish, or modify an immune response.

In another aspect, the invention provides a method of modulating or altering a T-cell response specific to an antigen in a subject, the method comprising administering to the subject at least one polynucleotide sequence encoding a polypeptide comprising any of SEQ ID NOS:48-94, 174-252, 263-272 and 283-293 or fragment thereof, and a

15   polynucleotide sequence encoding the antigen or antigenic fragment thereof, wherein each of the at least one polynucleotide sequences is expressed in the subject in an amount effective to modulate or alter a T cell response.

The invention also includes a method of modulating or altering an immune response in a subject, the method comprising introducing into cells of a tumor of the subject

20   at least one polynucleotide sequence encoding a polypeptide comprising any of SEQ ID NOS:48-94, 174-252, 263-272 and 283-293 or fragment thereof, wherein the polypeptide or fragment thereof interacts with or binds to a T cell receptor when expressed in a subject, and wherein the at least one polynucleotide sequence is operably linked to a promoter for expression in the subject and is present in an amount sufficient that when expressed is

25   effective to modulate or alter a T cell response.

In addition, the invention includes a vector comprising at least one polynucleotide sequence encoding a polypeptide comprising any of SEQ ID NOS:48-94, 174-252, 263-272 and 283-293 or fragment thereof, wherein the polypeptide or fragment thereof interacts with or binds to a T cell receptor when expressed in a subject, wherein the

30   at least one polynucleotide sequence is operably linked to a promoter for expression in the subject and is present in an amount sufficient that when expressed is effective to modulate or alter a T cell response.

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In another aspect, the invention provides vector comprising at least one polynucleotide sequence encoding a polypeptide comprising any of SEQ ID NOS:48-94, 174-252, 263-272 and 283-293 or fragment thereof, and a polynucleotide sequence encoding the antigen or antigenic fragment thereof, wherein the NCSM polypeptide or

5 fragment thereof interacts with or binds to a T cell receptor when expressed in a subject, and wherein each of the at least one polynucleotide sequences is operably linked to a promoter for expression in the subject and is present in an amount sufficient that when expressed is effective to modulate or alter a T cell response.

In general, nucleic acids and proteins derived by mutation, recursive

10 sequence recombination (RSR) or other alterations of the sequences herein are a feature of the invention. Similarly, those produced by recombination, including recursive sequence recombination, are a feature of the invention. Mutation and recombination methods using the nucleic acids described herein are a feature of the invention. For example, one method of the invention includes recombining one or more nucleic acids described herein with one

15 or more additional nucleic acids (including, but not limited to those noted herein), the additional nucleic acid encoding a NCSM polypeptide, co-stimulatory homologue or subsequence thereof. The recombining steps are optionally performed in vivo, ex vivo, or in vitro. Also included in the invention are a recombinant nucleic acid produced by this method, a cell containing the recombinant nucleic acid, a nucleic acid library produced by

20 this method comprising recombinant polynucleotides, and a population of cells containing the library comprising recombinant polynucleotides.

The invention also includes a method of designing or identifying agonists and antagonists of CD28 and CTLA-4 (which either enhance or inhibit signaling through CD28 or CTLA-4) based on the 3-dimensional structure of the polypeptides of the invention

25 (e.g., SEQ ID NOS:48-94, 174-252, 263-272, and 283-293).

The invention also includes soluble polypeptides and proteins (including fusion polypeptides and proteins) and nucleic acids encoding such soluble polypeptides and proteins. The invention also includes the use of such polypeptides and proteins as therapeutics, prophylactics, and diagnostics in therapeutic treatment and/or prevention of a

30 variety of diseases and conditions. Soluble polypeptides and proteins (and nucleic acids encoding them) include, e.g., extracellular domain (ECD) amino acid sequences of each NCSM (e.g., each CTLA-4 binding protein and CD28 binding protein) described herein (or fragments thereof) and nucleic acids encoding same, as well as constructs comprising, e.g.,

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each of said ECD, or fragments thereof, with an Ig polypeptide sequence (or fragment or variant thereof) (and nucleotide sequences encoding same) as fusion proteins. Some such soluble polypeptides and proteins exhibit a CD28/CTLA-4 binding affinity ratio that is greater than that of hB7-1 and/or have an ability to induce a T cell proliferation response, in the presence of activated T cells, that is less than that induced by soluble hB7-1 polypeptide in the presence of activate T cells.

In another aspect, the invention provides a computer or computer readable medium comprising a database comprising a sequence record comprising one or more character strings corresponding to a nucleic acid or protein sequence selected from any of SEQ ID NOS:1-272 and 283-293. The invention further includes an integrated system comprising a computer or computer readable medium comprising a database comprising one or more sequence records, each comprising one or more character strings corresponding to a nucleic acid or protein sequence selected from any of SEQ ID NOS:1-272 and 283-293, the integrated system further comprising a user input interface allowing a user to selectively view one or more sequence records. Also provided are methods of using a computer system to present information pertaining to at least one of a plurality of sequence records stored in a database, said sequence records each comprising one or more character strings corresponding to any of SEQ ID NOS:1-272 and 283-293.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a schematic representation of the following exemplary interactions: 1) interactions between a T cell receptor (TCR) and antigenic peptide presented in the groove of a major histocompatibility complex (MHC) molecule, and 2) interactions between a recombinant CD28BP polypeptide of the invention expressed on the surface of an antigen-presenting cell (APC) and a CD28 receptor on a T cell. Figure 1B is a schematic representation of exemplary interactions: 1) between a TCR and antigenic peptide presented in the groove of a MHC molecule, and 2) between a recombinant CTL4-BP polypeptide of the invention expressed on the surface of an APC and a CTLA-4 receptor on a T cell. The representation illustrates the principle by which recombinant polypeptides of the invention which preferentially bind the CD28 or CTLA-4 receptor effectuate enhanced or suppressed T cell activation.

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Figures 2A-2H depict an alignment of a naturally-occurring (*i.e.*, wild-type) human B7-1 polypeptide sequence (SEQ ID NO:278) and exemplary CD28BP polypeptide sequences of the invention (SEQ ID NOS:48-68, SEQ ID NOS:174-221, and SEQ ID NO:283). The predicted boundaries between the signal peptide region, extracellular domain (ECD), transmembrane domain (TMD), and cytoplasmic domain (CD), based on corresponding boundaries in the hB7-1 sequence are shown at the top. SEQ ID NO:283 represents a "consensus sequence" of these aligned CD28BP sequences. The arrow positioned between the amino acid residues equivalent to amino acid residues 34-35 of SEQ ID NO:278, indicates the predicted boundary between the signal peptide region and the mature polypeptide region based on comparison with the hB7-1 sequence.

Figures 3A-3H illustrate an alignment of a naturally-occurring (*i.e.*, wild-type) hB7-1 polypeptide sequence (SEQ ID NO: 278) and exemplary CTLA-4BP polypeptide sequences of the invention (SEQ ID NOS: 69-73, SEQ ID NOS:74-92, SEQ ID NO:222-252, and SEQ ID NO:286). The predicted boundaries between the signal peptide sequence, ECD, TMD, and CD, based on corresponding boundaries in the hB7-1 sequence are shown at the top.

SEQ ID NO:286 represents a "consensus sequence" of these aligned CTLA-4BP sequences of the invention. Alignments shown in Figures 2A-2H and 3A-3H were prepared using the CLUSTALW multiple sequence alignment program, a part of the Vector NTI version 6 sequence analysis software package (Informax, Bethesda, MD). CLUSTALW initially performs multiple pairwise comparisons between groups of sequences and then assembles the pairwise alignments into a multiple alignment based on homology. For the initial pairwise alignments, Gap Open and Gap Extension penalties were 10 and 0.1, respectively. For the multiple alignments, Gap Open penalty was 10, and the Gap Extension penalty was 0.05. The BLOSUM62 matrix was the protein weight matrix.

Figures 4A-4D presents graphs illustrating competitive FACS binding profiles of hB7-1, clone CD28BP-15, clone CTLA-4BP 5x4-12c, and vector control for each of soluble CD28-Ig receptor and soluble CTLA-4-Ig receptor.

Figure 5 presents graphs depicting competitive FACS binding profiles of seventeen Round 2 CD28BP clones for each of soluble CD28-Ig receptor and soluble CTLA-4-Ig receptor.

Figure 6A is a schematic representation of an exemplary competitive FACS binding profile for a CTLA-4BP clone for soluble CD28-Ig receptor and soluble CTLA-4-Ig

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receptor. Figure 6B is a schematic representation of an exemplary competitive FACS binding profile for a CD284BP clone for soluble CD28-Ig receptor and soluble CTLA-4-Ig receptor.

Figures 7A-7H are graphs showing competitive FACS binding profiles of WT human B7-1 (CD80), five CTLA-4BP clones, and HEK 293 cells (control) for soluble CD28-Ig receptor and soluble CTLA-4-Ig receptor.

Figures 8A-8B present schematic representations of the amino acid sequences of CD28BP-12 and CTLA-4BP 5x4-12c and the genealogy of these sequences.

Figures 9A-9F are graphs depicting the mean fluorescence intensities generated by the binding of labeled soluble ligand sCD28-Ig and labeled soluble ligand sCTLA4-Ig to clones CD28BP-15 and CTLA-4BP 5x4-12c. Figures 9G-9H provide graphs illustrating histograms from the staining of stable 293 transfectants expressing CTLA-4BP 5x4-12c (gray histograms), hB7-1 (gray histograms) and negative control transfectants (open histograms) with anti-hB7-1 monoclonal antibodies (mAbs) with expression levels analyzed by flow cytometry.

Figure 10 shows a graph depicting T cell proliferation response, as measured by ^{3}H thymidine incorporation, resulting from the co-culturing of cells transfected with one of seventeen CD28BP clones, human B7-1 (CD80), or an empty control vector cultured with anti-CD3 mAbs.

Figures 11A-11C present graphs illustrating improved co-stimulation of purified human T cells observed co-culturing irradiated 293 cells transiently (A) or stably (B) transfected with clone CD28BP-15, hB7-1, or a control vector with purified T cells and anti-CD3 mAbs. Figure 11C shows a graph depicting levels of IFN-gamma produced by co-culturing irradiated stable transfectants expressing CD28BP or hB7-1 or negative control cells transfected with an "empty" vector with purified human T cells.

Figure 12 shows a graph depicting T cell proliferation response, as measured by ^{3}H thymidine incorporation, resulting from the co-culturing of cells transfected with one of nineteen CTLA-4BP clones, WT human B7-1 (CD80), or an empty control vector cultured with soluble anti-CD3 mAbs.

Figures 13A-13D show graphs illustrating the effects of cells transfected with clone CTLA-4 BP 5x4-12C, hB7-1 or a control vector cultured on T cell proliferation induced by co-culturing the transfectants with purified T cells in the presence of soluble anti-CD3 mAbs and on cytokine synthesis in mixed lymphocyte reaction assay.

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Figures 14A-14B are schematic representations of exemplary soluble forms of human B7-1 molecules. Expression plasmids were constructed by juxtaposing the nucleotide sequence encoding a signal sequence and extracellular domain (ECD) (or ECD fragment) of WT hB7-1 with a nucleotide sequence encoding E epitope and/or His Tag or

5   human Ig Fc domain to create a IgG fusion protein. Figure 14A shows a representation of a fusion protein expressed by one such plasmid comprising a soluble WT human B7-1-ECD, including a signal sequence peptide (amino acid residues 1-34), ECD (amino acid residues 35-242), and E-epitope tag (amino acid residues 243-259) and His-tag (amino acid residues 260-268). Numbering coincides with the ATG or Met. The amino acid residues positioned

10   at the beginning and end of an exemplary ECD amino acid sequence are shown. Figure 14B is an illustration of a fusion protein expressed by one such plasmid comprising a soluble WT hB7-1-ECD-Ig fusion protein, including the signal domain (amino acid residues 1-34), ECD domain (amino acid residues 35-242), Factor Xa (IGER), valine-threonine (VT) or (BsetII) glycine-valine-threonine (GVT) linker, and hinge CH2-CH3 (constant/heavy)

15   region of the Fc domain of IgG1 (e.g., GenBank Access. No. P01857 or X70421) (showing the initial amino acid residues corresponding to nucleic acid sequence shown at GenBank Accession No. X70421). The amino acid residues positioned at the beginning and end of an exemplary ECD amino acid sequence are shown. Optionally, other Ig molecules, or Ig Fc fragments thereof, can used to construct NCSM-Ig fusion proteins. Similar expression

20   plasmids were constructed by substituting a nucleotide sequence encoding a NCSM polypeptide of the invention for the sequence encoding the hB7-1 ECD domain, and fusion proteins comprising NCSM-ECD sequences were generated from such plasmids. A nucleotide sequence encoding truncated ECD domain of hB7-1 or a NCSM polypeptide can also be substituted. The signal sequence may be the WT hB7-1 signal sequence or a

25   recombinant signal sequence from a recombinant NCSM polynucleotide. The B7-1- or NCSM-ECD-Ig fusion protein may include Factor Xa cleavage site.

Figure 15 illustrates an example of a pNCSMsECD plasmid expression vector comprising a nucleotide sequence encoding a soluble extracellular domain of a NCSM polypeptide of the invention with an E-epitope tag and/or histidine tag.

30   Figure 16 is a photographic representation of a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of various soluble forms of WT B7-1 (ECD and fusion protein and delta Cys mutant) and clone CD28BP-15. Molecular weight standards are shown on the left for comparison: myosin (185 kDa); phosphorylase B

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(98 kDa); glutamic dehydrogenase (52 kDa); carbonic anhydrase (31 kDa); myoglobin (19 kDa); and lysozyme (11 kDa).

Figure 17 illustrates an example of a phB7-1ECD-Ig plasmid expression vector comprising a nucleotide sequence encoding a soluble extracellular domain of a human B&-1/IgG1 Fc domain fusion protein. A nucleotide sequence encoding the extracellular domain of a NCSM polypeptide (or fragment thereof) can be substituted for the human B7-1-ECD sequence.

Figure 18 is a photographic representation of an SDS-PAGE gel analysis of affinity purified CD28BP-15 ECD-Ig, CTLA-4BP 5X4-12C ECD-Ig, and WT human B7-1 ECD-Ig fusion proteins. Molecular weight standards are shown on the left.

Figure 19 is a photograph of a Western blot analysis.

Figures 20A and 20E are graphs depicting T cell proliferation responses (measured via ^{3}H thymidine uptake (counts per minute (CPM)) generated by co-culturing various crosslinked (multimeric) soluble NCSM-ECD fusion proteins and hB7-1-ECD fusion proteins with purified T cells in the presence of soluble anti-CD3 mAbs. Figure 20B is a graph depicting T cell proliferation responses generated by co-culturing various non-crosslinked soluble NCSM-ECD with peripheral blood mononuclear cells (PBMCs). Figures 20C and 20D are graphs depicting T cell proliferation responses induced by co-culturing various non-crosslinked soluble NCSM-ECDs with PBMC and phytohemagglutinin (PHA). Figure 20F is a graph depicting proliferation of T cells, measuring ^{3}H thymidine uptake in a mixed lymphocyte reaction.

Figure 21 illustrates an exemplary pMaxVax10.1 plasmid expression vector.

Figure 22A illustrates an exemplary pMaxVax10.1 plasmid expression vector that comprises a nucleotide sequence encoding a CD28BP polypeptide. Figure 22B illustrates a bicistronic pMaxVax10.1 plasmid expression vector that comprises a nucleotide sequence encoding a CD28BP polypeptide and a nucleotide sequence encoding the cancer antigen EpCam/KSA. Positions of various components of the vectors, including the promoter(s), kanamycin resistant gene, ColE1 replication of origin, BGH poly A adenylation sequences and restriction sites are shown.

Figures 23A-23B are histograms depicting the binding of full-length hB7-1-Tyr65His variant, CTLA-4BP 5x4-12c (gray histogram), and hB7-1 (gray histogram) to either soluble CD28-Ig or CTLA-4-Ig.

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Figure 24 shows a T cell proliferation assay (^{3}H thymidine uptake measured in counts per minute) using 293 HEK cells transfected with pCDNA or with a nucleic acid sequence encoding full-length hB7-1 (SEQ ID NO:278), hB7-1-Tyr65His polypeptide variant, full-length CTLA-4BP 5x4-12c (SEQ ID NO:39) (clone 12c), or full-length

5   CD28BP-15 (SEQ ID NO:19); 293 cells alone (with no DNA) were also assayed for their ability to induce human T-cell proliferation.

Figures 25A-25C are graphs depicting T cell proliferation responses generated by co-culturing PBMCs, tetanus toxoid (antigen) and increasing concentrations of soluble proteins of non-crosslinked CD28BP-15-ECD-Ig, CD28BP-15-ECD, WT huB7.1-

10   ECD-Ig, WT huB7.1-ECD, commercial CTLA-4-Ig receptor/ligand (R&D Systems), and control human IgG antibody.

DETAILED DESCRIPTION OF THE INVENTION

The expression of B7-1 has been shown to be an important mechanism of immune responses in mammals, including humans. It is believed that at least two signals

15   are required for activation of T cells by antigen-bearing target cells: 1) an antigen-specific signal, delivered through the T cell receptor (TCR); and 2) an antigen-independent or co-stimulatory signal that leads to the production of lymphokine products (Hodge et al. (1994) Cancer Res. 54:5552-5555). B7-1, which is typically expressed on antigen-presenting cells (APC), has been determined to be a ligand for two T cell surface antigen receptors: CD28

20   and CTLA-4. Both receptors are present on T cells, although they are expressed at different times and in different amounts. T cell activation is a prerequisite for all specific immune responses. However, if only one T cell activation signal is received by a T cell, activation will likely not occur, and anergy may result. For example, many tumor cells do not express B7-1. Consequently, even when a tumor cell expresses a potential rejection antigen, it is

25   not likely that it will be able to activate an antitumor T cell response. Id. For T cell activation and enhanced immune response, an additional antigen-independent signal, such as from B7-1, is believed necessary.

The human CD28 receptor and human CTLA-4 receptor are naturally activated in human cells by B7-1. In some studies, the reported binding affinities of CTLA-

30   4 and CD28 to WT hB7-1 were found to be about $0.2-0.4 \times 10^{-6}$ M and about 4×10^{-6} M, respectively (van der Merwe et al. (1997) J. Exp. Med. 185:393; Ikemizu et al. (2000) Immunity 12:51). However, different studies have reported different binding affinities.

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The amino acid sequence of full-length WT hB7-1 comprises 288 amino acids (GenBank Protein Access. No. P33681) (SEQ ID NO:278). The signal peptide of WT hB7-1 (which is cleaved in the secreted form) typically comprises amino acid residues 1-34, the extracellular domain (ECD) comprises amino acid residues 35-242, the transmembrane domain comprises amino acid residues 243-263, and the cytoplasmic domain comprises amino acid residues 264-288. The mature form of hB7-1, which has a total of 254 amino acids, comprises amino acid residues 35-288 (the full-length sequence without the signal peptide), and begins with the amino acid sequence: valine-isoleucine-histidine-valine. If desired, the amino acids of the mature form can be numbered beginning with the Val of the Val-Ile-His-Val sequence, designating Val as the first residue (e.g., the ECD comprises amino acid residues numbered 1-208). In another aspect, the ECD of hB7-1 comprises amino acid residues 1-208, the transmembrane domain comprises amino acid residues 209-235, and the cytoplasmic domain comprises amino acid residues 236-254 of the full-length mature hB7-1 sequence when numbered beginning with the Val of the Val-Ile-His-Val sequence of the mature sequence as described above. See, e.g., US 6,071,716. There are eight possible glycosylation sites (Asn-X-Ser/Thr) in hB7-1 ECD. The transmembrane domain includes at least 3 cysteine residues that may be involved in binding to other polypeptides or lipid derivatization. Id.

In yet another aspect, for hB7-1, the ECD comprises amino acid residues 35-251, the TMD comprises amino acid residues 252-267, and the CD comprises amino acid residues 268-288. From the full-length NCSM polypeptides set forth herein, the subsequences corresponding to the signal peptide, ECD, TMD, and CD of the NCSM polypeptide, respectively, can be determined as described in greater detail below by, e.g., alignment of the NCSM amino acid sequence with hB7-1 amino acid sequence or by using known methods to predict the location and/or cleavage sites of such sequences (e.g., methods to predict signal peptide cleavage sites, computer program to determine, e.g., regions of hydrophobicity corresponding to the amino acid residues of a TMD, etc.).According to one study, the nucleic acid sequence of WT hB7-1 comprises 1491 base pairs and is set forth in US Pat. No. 6,071,716 (see SEQ ID NO:1 therein). An alignment of the hB7-1 nucleic acid sequence with its corresponding full-length amino acid sequence is also shown in US Pat. No. 6,071,716 (see SEQ ID NO:1 therein).

Using the nucleotide sequences of human B7-1 and other selected mammalian B7-1 molecules, we generated recombination nucleotides encoding

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recombinant chimeric co-stimulatory peptides molecules having altered properties as compared those of WT hB7. This embodiment and others are described in detail below. Figure 1 illustrates an interaction between an NCSM molecule of the invention, as expressed of an APC cell, and corresponding receptor, expressed on a T cell.

5   DEFINITIONS

Unless otherwise defined herein or below in the remainder of the specification, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs.

A "polynucleotide sequence" is a nucleic acid which comprises a polymer of
10   nucleic acid residues or nucleotides (A,C,T,U,G, etc. or naturally occurring or artificial nucleotide analogues), or a character string representing a nucleic acid, depending on context. Either the given nucleic acid or the complementary nucleic acid can be determined from any specified polynucleotide sequence.

A "polypeptide sequence" is a polymer of amino acids (a protein,
15   polypeptide, etc., comprising amino acid residues) or a character string representing an amino acid polymer, depending on context. Given the degeneracy of the genetic code, one or more nucleic acids, or the complementary nucleic acids thereof, that encode a specific polypeptide sequence can be determined from the polypeptide sequence.

A nucleic acid, protein, peptide, polypeptide, or other component is
20   "isolated" when it is partially or completely separated from components with which it is normally associated (other peptides, polypeptides, proteins (including complexes, e.g., polymerases and ribosomes which may accompany a native sequence), nucleic acids, cells, synthetic reagents, cellular contaminants, cellular components, etc.), e.g., such as from other components with which it is normally associated in the cell from which it was originally
25   derived. A nucleic acid, polypeptide, or other component is isolated when it is partially or completely recovered or separated from other components of its natural environment such that it is the predominant species present in a composition, mixture, or collection of components (i.e., on a molar basis it is more abundant than any other individual species in the composition). In preferred embodiments, the preparation consists of more than about
30   70% or 75%, typically more than about 80%, or preferably more than about 90% of the isolated species.

In one aspect, a "substantially pure" or "isolated" nucleic acid (e.g., RNA or DNA), polypeptide, protein, or composition also means where the object species (e.g., nucleic acid or polypeptide) comprises at least about 50, 60, or 70 percent by weight (on a molar basis) of all macromolecular species present. A substantially pure or isolated

5   composition can also comprise at least about 80, 90, or 95 percent by weight of all macromolecular species present in the composition. An isolated object species can also be purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of derivatives of a single macromolecular species. The term "purified" generally

10   denotes that a nucleic acid, polypeptide, or protein gives rise to essentially one band in an electrophoretic gel. It typically means that the nucleic acid, polypeptide, or protein is at least about 50% pure, 60% pure, 70% pure, 75% pure, more preferably at least about 85% pure, and most preferably at least about 99% pure.

The term "isolated nucleic acid" may refer to a nucleic acid (e.g., DNA or

15   RNA) that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (i.e., one at the 5' and one at the 3' end) in the naturally occurring genome of the organism from which the nucleic acid of the invention is derived. Thus, this term includes, e.g., a cDNA or a genomic DNA fragment produced by polymerase chain reaction (PCR) or restriction endonuclease treatment, whether such cDNA or genomic DNA

20   fragment is incorporated into a vector, integrated into the genome of the same or a different species than the organism, including, e.g., a virus, from which it was originally derived, linked to an additional coding sequence to form a hybrid gene encoding a chimeric polypeptide, or independent of any other DNA sequences. The DNA may be double-stranded or single-stranded, sense or antisense.

25   The term "recombinant" when used with reference, e.g., to a cell, nucleotide, vector, protein, or polypeptide typically indicates that the cell, nucleotide, or vector has been modified by the introduction of a heterologous (or foreign) nucleic acid or the alteration of a native nucleic acid, or that the protein or polypeptide has been modified by the introduction of a heterologous amino acid, or that the cell is derived from a cell so

30   modified. Recombinant cells express nucleic acid sequences (e.g., genes) that are not found in the native (non-recombinant) form of the cell or express native nucleic acid sequences (e.g., genes) that would be abnormally expressed under-expressed, or not expressed at all. The term "recombinant" when used with reference to a cell indicates that the cell replicates

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a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. Recombinant cells can contain genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also contain genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell by

5   artificial means. The term also encompasses cells that contain a nucleic acid endogenous to the cell that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques.

A "recombinant polynucleotide" or a "recombinant polypeptide" is a non-

10   naturally occurring polynucleotide or polypeptide that includes nucleic acid or amino acid sequences, respectively, from more than one source nucleic acid or polypeptide, which source nucleic acid or polypeptide can be a naturally occurring nucleic acid or polypeptide, or can itself have been subjected to mutagenesis or other type of modification. A nucleic acid or polypeptide may be deemed "recombinant" when it is artificial or engineered, or

15   derived from an artificial or engineered polypeptide or nucleic acid. A recombinant nucleic acid (e.g., DNA or RNA) can be made by the combination (e.g., artificial combination) of at least two segments of sequence that are not typically included together, not typically associated with one another, or are otherwise typically separated from one another. A recombinant nucleic acid can comprise a nucleic acid molecule formed by the joining

20   together or combination of nucleic acid segments from different sources and/or artificially synthesized. A "recombinant polypeptide" (or "recombinant protein") often refers to a polypeptide (or protein) that results from a cloned or recombinant nucleic acid or gene. The source polynucleotides or polypeptides from which the different nucleic acid or amino acid sequences are derived are sometimes homologous (i.e., have, or encode a polypeptide that

25   encodes, the same or a similar structure and/or function), and are often from different isolates, serotypes, strains, species, of organism or from different disease states, for example.

The term "recombinantly produced" refers to an artificial combination usually accomplished by either chemical synthesis means, recursive sequence

30   recombination of nucleic acid segments or other diversity generation methods (such as, e.g., shuffling) of nucleotides, or manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques known to those of ordinary skill in the art. "Recombinantly expressed" typically refers to techniques for the production of a recombinant nucleic acid in

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vitro and transfer of the recombinant nucleic acid into cells in vivo, in vitro, or ex vivo where it may be expressed or propagated.

A "recombinant expression cassette" or simply an "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with nucleic acid elements that are capable of effecting expression of a structural gene in hosts compatible with such sequences. Expression cassettes include at least promoters and optionally, transcription termination signals. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (e.g., a nucleic acid encoding a desired polypeptide), and a promoter. Additional factors necessary or helpful in effecting expression may also be used as described herein. For example, an expression cassette can also include nucleotide sequences that encode a signal sequence that directs secretion of an expressed protein from the host cell. Transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

By "immune response" is intended an alteration of an organism's or subject's immune system in response to an immunomodulatory agent, immunogen, or antigen that may include, but is not limited to, antibody production, induction of cell-mediated immunity, complement activation, development of immunological tolerance, inhibition of an immune response, or breaking of immunological tolerance. An "immunomodulatory agent" or "immunomodulatory molecule" modulates an immune response. An "immunogen" refers generally to a substance capable of provoking or altering an immune response, and includes, but is not limited to, e.g., immunogenic proteins, polypeptides, and peptides; antigens and antigenic peptide fragments thereof; and nucleic acids having immunogenic properties or encoding, e.g., polypeptides having such properties.

An "immunogen" refers to a substance capable of provoking an immune response, and includes, e.g., antigens, autoantigens that play a role in induction of autoimmune diseases, and tumor-associated antigens expressed on cancer cells. An immune response generally refers to the development of a cellular or antibody-mediated response to an agent, such as an antigen or fragment thereof or nucleic acid encoding such agent. In some instances, such a response comprises a production of at least one or a combination of CTLs, B cells, or various classes of T cells that are directed specifically to antigen-presenting cells expressing the antigen of interest.

By "modulation" or "modulating" an immune response of a subject is intended that the immune response of the subject is altered. For example, "modulation" or

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"modulating" an immune response of a subject means, e.g., that the immune response is stimulated, invoked, decreased, increased, enhanced, or otherwise altered. The immunological response may be skewed or shifted from Th1 to Th2 or vice versa to optimize protection and reduce unwanted side effects of the immunological response. An

5   immunomodulatory agent or molecule modulates an immune response. An immunomodulatory agent has an immunomodulatory activity. A modulated immune response of a subject to whom an immunomodulatory agent is administered differs from the immune response of an untreated subject to whom the immunomodulatory has not been administered by 0.1%, 0.5%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%,

10   100%, or more. Modulation of an immune response in a subject can be assessed by means known to those skilled in the art, including those described below.

"Tolerance" refers to a state of diminished or lack of immunological responsiveness. Tolerance typically defines an absent or diminished or lessened capacity of a subject to mount an immune response against a given antigen, usually the result of, e.g.,

15   contact between the subject and a target antigen under non-immunizing conditions.

"Anergy" refers to a state of diminished reactivity to one or more antigens. For example, anergy state is often characterized by diminished T cell responses, e.g., proliferation or IL-2 production, when specific T cells are restimulated under otherwise stimulatory conditions.

20   An "antigen" refers to a substance that is capable of inducing an immune response (e.g., humoral and/or cell-mediated) in a host, including, but not limited to, eliciting the formation of antibodies in a host, or generating a specific population of lymphocytes reactive with that substance. Antigens are typically macromolecules (e.g., proteins and polysaccharides) that are foreign to the host.

25   A "subsequence" or "fragment" is any portion of an entire sequence, up to and including the complete sequence. Thus, a "subsequence" refers to a sequence of nucleic acids or amino acids that comprises a part of a longer sequence of nucleic acids (e.g., polynucleotide) or amino acids (e.g., polypeptide) respectively.

An "adjuvant" refers to a substance that enhances an immune response,

30   including, for example, but not limited to, an antigen's immune-stimulating properties or the pharmacological effect(s) of a compound or drug. An adjuvant may non-specifically enhance an immune response, e.g., the immune response to an antigen. "Freund's Complete Adjuvant," for example, is an emulsion of oil and water containing an immunogen, an

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emulsifying agent and mycobacteria. Another example, "Freund's incomplete adjuvant," is the same, but without mycobacteria. An adjuvant may comprise oils, emulsifiers, killed bacteria, aluminum hydroxide, or calcium phosphate (e.g., in gel form), or combinations thereof. An adjuvant may be administered into a subject (e.g., via injection intramuscularly

5   or subcutaneously) in an amount sufficient to produce antibodies.

Numbering of a given amino acid polymer or nucleotide polymer "corresponds to numbering" of a selected amino acid polymer or nucleic acid polymer when the position of any given polymer component (e.g., amino acid residue, nucleotide residue) is designated by reference to the same or an equivalent residue position in the selected

10   amino acid or nucleotide polymer, rather than by the actual position of the component in the given polymer. Thus, for example, the numbering of a given amino acid position in a given polypeptide sequence corresponds to the same or equivalent amino acid position in a selected polypeptide sequence used as a reference sequence.

A vector is a component or composition for facilitating cell transduction or

15   transfection by a selected nucleic acid, or expression of the nucleic acid in the cell. Vectors include, e.g., plasmids, cosmids, viruses, YACs, bacteria, poly-lysine, etc. An "expression vector" is a nucleic acid construct or sequence, generated recombinantly or synthetically, with a series of specific nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic

20   acid fragment. The expression vector typically includes a nucleic acid to be transcribed operably linked to a promoter. The nucleic acid to be transcribed is typically under the direction or control of the promoter.

"Substantially the entire length of a polynucleotide sequence" or "substantially the entire length of a polypeptide sequence" refers to at least about 50%,

25   generally at least about 60%, 70%, or 75%, usually at least about 80%, or typically at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more of a length of a polynucleotide sequence or polypeptide sequence.

"Naturally occurring" as applied to an object refers to the fact that the object can be found in nature as distinct from being artificially produced by man. A polypeptide or

30   polynucleotide sequence that is present in an organism (including viruses, bacteria, protozoa, insects, plants or mammalian tissue) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring. Non-naturally occurring as applied to an object means that the object is not

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naturally-occurring -- i.e., the object cannot be found in nature as distinct from being artificially produced by man.

The term "immunoassay" includes an assay that uses an antibody or immunogen to bind or specifically bind an antigen. The immunoassay is typically

5 characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

The term "homology" generally refers to the degree of similarity between two or more structures. The term "homologous sequences" refers to regions in macromolecules that have a similar order of monomers. When used in relation to nucleic

10 acid sequences, the term "homology" refers to the degree of similarity between two or more nucleic acid sequences (e.g., genes) or fragments thereof. Typically, the degree of similarity between two or more nucleic acid sequences refers to the degree of similarity of the composition, order, or arrangement of two or more nucleotide bases (or other genotypic feature) of the two or more nucleic acid sequences. The term "homologous nucleic acids"

15 generally refers to nucleic acids comprising nucleotide sequences having a degree of similarity in nucleotide base composition, arrangement, or order. The two or more nucleic acids may be of the same or different species or group. The term "percent homology" when used in relation to nucleic acid sequences, refers generally to a percent degree of similarity between the nucleotide sequences of two or more nucleic acids.

20 When used in relation to polypeptide (or protein) sequences, the term "homology" refers to the degree of similarity between two or more polypeptide (or protein) sequences (e.g., genes) or fragments thereof. Typically, the degree of similarity between two or more polypeptide (or protein) sequences refers to the degree of similarity of the composition, order, or arrangement of two or more amino acid of the two or more

25 polypeptides (or proteins). The two or more polypeptides (or proteins) may be of the same or different species or group. The term "percent homology" when used in relation to polypeptide (or protein) sequences, refers generally to a percent degree of similarity between the amino acid sequences of two or more polypeptide (or protein) sequences. The term "homologous polypeptides" or "homologous proteins" generally refers to polypeptides

30 or proteins, respectively, that have amino acid sequences and functions that are similar. Such homologous polypeptides or proteins may be related by having amino acid sequences and functions that are similar, but are derived or evolved from different or the same species using the techniques described herein.

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The term "subject" as used herein includes, but is not limited to, an organism; a mammal, including, e.g., a human, non-human primate (e.g., baboon, orangutan, monkey), mouse, pig, cow, goat, cat, rabbit, rat, guinea pig, hamster, horse, monkey, sheep, or other non-human mammal; a non-mammal, including, e.g., a non-

5   mammalian vertebrate, such as a bird (e.g., a chicken or duck) or a fish, and a non-mammalian invertebrate.

The term "pharmaceutical composition" means a composition suitable for pharmaceutical use in a subject, including an animal or human. A pharmaceutical composition generally comprises an effective amount of an active agent and a carrier,

10   including, e.g., a pharmaceutically acceptable carrier.

The term "effective amount" means a dosage or amount sufficient to produce a desired result. The desired result may comprise an objective or subjective improvement in the recipient of the dosage or amount.

A "prophylactic treatment" is a treatment administered to a subject who does

15   not display signs or symptoms of a disease, pathology, or medical disorder, or displays only early signs or symptoms of a disease, pathology, or disorder, such that treatment is administered for the purpose of diminishing, preventing, or decreasing the risk of developing the disease, pathology, or medical disorder. A prophylactic treatment functions as a preventative treatment against a disease or disorder. A "prophylactic activity" is an

20   activity of an agent, such as a nucleic acid, vector, gene, polypeptide, protein, substance, or composition thereof that, when administered to a subject who does not display signs or symptoms of pathology, disease or disorder, or who displays only early signs or symptoms of pathology, disease, or disorder, diminishes, prevents, or decreases the risk of the subject developing a pathology, disease, or disorder. A "prophylactically useful" agent or

25   compound (e.g., nucleic acid or polypeptide) refers to an agent or compound that is useful in diminishing, preventing, treating, or decreasing development of pathology, disease or disorder.

A "therapeutic treatment" is a treatment administered to a subject who displays symptoms or signs of pathology, disease, or disorder, in which treatment is

30   administered to the subject for the purpose of diminishing or eliminating those signs or symptoms of pathology, disease, or disorder. A "therapeutic activity" is an activity of an agent, such as a nucleic acid, vector, gene, polypeptide, protein, substance, or composition thereof, that eliminates or diminishes signs or symptoms of pathology, disease or disorder,

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when administered to a subject suffering from such signs or symptoms. A "therapeutically useful" agent or compound (e.g., nucleic acid or polypeptide) indicates that an agent or compound is useful in diminishing, treating, or eliminating such signs or symptoms of a pathology, disease or disorder.

5   The term "gene" broadly refers to any segment of DNA associated with a biological function. Genes include coding sequences and/or regulatory sequences required for their expression. Genes also include non-expressed DNA nucleic acid segments that, e.g., form recognition sequences for other proteins (e.g., promoter, enhancer, or other regulatory regions). Genes can be obtained from a variety of sources, including cloning
10 from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

        Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, molecular biology, nucleic acid chemistry, and protein chemistry described below are those well known and commonly employed by those of
15 ordinary skill in the art. Standard techniques, such as described in Sambrook et al., Molecular Cloning - A Laboratory Manual (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 (hereinafter "Sambrook") and Current Protocols in Molecular Biology, F. M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994,
20 supplemented through 1999) (hereinafter "Ausubel"), are used for recombinant nucleic acid methods, nucleic acid synthesis, cell culture methods, and transgene incorporation, e.g., electroporation, injection, gene gun, impressing through the skin, and lipofection. Generally, oligonucleotide synthesis and purification steps are performed according to specifications. The techniques and procedures are generally performed according to
25 conventional methods in the art and various general references which are provided throughout this document. The procedures therein are believed to be well known to those of ordinary skill in the art and are provided for the convenience of the reader.

        As used herein, an "antibody" refers to a protein comprising one or more polypeptides substantially or partially encoded by immunoglobulin genes or fragments of
30 immunoglobulin genes. The term antibody is used to mean whole antibodies and binding fragments thereof. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or

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lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. A typical immunoglobulin (e.g., antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about

5   25 KDa) and one "heavy" chain (about 50-70 KDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains, respectively.

Antibodies exist as intact immunoglobulins or as a number of well

10   characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the (Fab')2 dimer into an Fab' monomer. The Fab'

15   monomer is essentially an Fab with part of the hinge region. The Fc portion of the antibody molecule corresponds largely to the constant region of the immunoglobulin heavy chain, and is responsible for the antibody's effector function (see, Fundamental Immunology, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an

20   intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies.

25   Antibodies also include single-armed composite monoclonal antibodies, single chain antibodies, including single chain Fv (sFv) antibodies in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide, as well as diabodies, tribodies, and tetrabodies (Pack et al. (1995) J Mol Biol 246:28; Biotechnol 11:1271; and Biochemistry 31:1579). The

30   antibodies are, *e.g.*, polyclonal, monoclonal, chimeric, humanized, single chain, Fab fragments, fragments produced by an Fab expression library, or the like.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of

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molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

5   An "antigen-binding fragment" of an antibody is a peptide or polypeptide fragment of the antibody that binds an antigen. An antigen-binding site is formed by those amino acids of the antibody that contribute to, are involved in, or affect the binding of the antigen. See Scott, T.A. and Mercer, E.I., Concise Encyclopedia: Biochemistry and Molecular Biology (de Gruyter, 3d ed. 1997), and Watson, J.D. et al., Recombinant DNA

10   (2d ed. 1992) [hereinafter "Watson, Recombinant DNA"], each of which is incorporated herein by reference in its entirety for all purposes.

The term "screening" describes, in general, a process that identifies optimal molecules of the present invention, such as, e.g., the NCSM polypeptide and proteins, fragments and homologues thereof, and related fusion polypeptides and proteins including

15   the same, nucleic acids encoding all such molecules. Several properties of these respective molecules can be used in selection and screening, for example, an ability of a respective molecule to bind to a receptor, to alter an immune response, e.g., induce or inhibit a desired immune response, in a test system or an in vitro, ex vivo or in vivo application (e.g., induce or inhibit a T cell proliferation response in conjunction with costimulation of T cell

20   receptor/CD3 (by, e.g., an antigen or anti-CD3 antibody)), or to bind a first receptor with equal, greater, or less binding affinity relative to a second receptor compared to the binding affinity of a control molecule (e.g., a wild-type B7-1 or co-stimulatory molecule) for the first and second receptors, as measured by the respective molecule's first receptor/second receptor binding affinity ratio (or its reciprocal), compared to the control molecule's first

25   receptor/second receptor binding affinity ratio. In the case of antigens, several properties of the antigen can be used in selection and screening including antigen expression, folding, stability, immunogenicity and presence of epitopes from several related antigens. Selection is a form of screening in which identification and physical separation are achieved simultaneously by expression of a selection marker, which, in some genetic circumstances,

30   allows cells expressing the marker to survive while other cells die (or vice versa). Screening markers include, for example, luciferase, beta-galactosidase and green fluorescent protein, and the like. Selection markers include drug and toxin resistance genes, and the like. Because of limitations in studying primary immune responses in vitro, in vivo

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or *ex vivo* studies are particularly useful screening methods. In these studies, genetic vaccines or expression vectors that include sequences encoding one or more respective NCSM polypeptides, are first introduced to test animals, and the immune responses are subsequently studied by analyzing protective immune responses or by studying the quality

5   or strength of the induced immune response using lymphoid cells derived from the immunized animal. Alternatively, the NCSM polypeptide itself or a soluble form thereof (e.g., the ECD of the polypeptide or a fragment thereof alone or in a fusion protein) is introduced to the test animal. Although spontaneous selection can and does occur in the course of natural evolution, in the present methods selection is performed by man.

10   A "specific binding affinity" between two molecules, e.g., a ligand and a receptor, means a preferential binding of one molecule for another in a mixture of molecules. The binding of the molecules is typically considered specific if the binding affinity is about 1×10^2 M^{-1} to about 1×10^7 M^{-1} (i.e., about 10^{-2} - 10^{-7} M) or greater.

         An "binding affinity ratio" refers to a relative ratio of the binding affinity of

15   a molecule of interest (e.g., a recombinant ligand, such as a NSCM polypeptide) for a first molecule (e.g., a first receptor, such as CD28 receptor) to the binding affinity of the same molecule of interest to a second molecule (e.g., a second receptor, such as CTLA-4 receptor). In one aspect, the relative binding affinity ratio may be determined by visual inspection, such as by, e.g., examining a FACS binding profile that displays the binding

20   affinity profile of the molecule of interest to both receptors, and evaluating the degree of relative binding of the molecule of interest to each of the first and second receptors. The results of this determination can be compared with a similar examination and evaluation of a FACS binding affinity profile displaying the binding affinity of a control molecule (e.g., wild-type ligand, such as a WT human, primate, or mammalian B7-1) to both receptors,

25   wherein the degree of relative binding of the control molecule to each of the receptors is evaluated. These and other procedures described below can be used to determine a CD28/CTLA-4 binding affinity ratio for a CD28BP polypeptide of the present invention and a CTLA-4/CD28 binding affinity ratio for a CTLA-4BP polypeptide of the present invention. Alternatively, a binding affinity ratio can be determined by making a ratio

30   between a quantitative measurement of the binding affinity of the molecule of interest (e.g., ligand) for the first receptor and a quantitative measurement of the binding affinity of the molecule of interest for the second receptor using known procedures for measuring binding

affinities. For example, known methods for measuring the binding affinity of human (or other mammalian) B7-1 for each of CD28 and CTLA-4 receptors can be used.

An "exogenous" nucleic acid," "exogenous DNA segment," "heterologous sequence," or "heterologous nucleic acid," as used herein, is one that originates from a
5   source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell, but has been modified. Modification of a heterologous sequence in the applications described herein typically occurs through the use of recursive sequence recombination. The terms refer to a DNA segment which is foreign or heterologous to the
10   cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited,
15   the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences and as well as the
20   sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer <u>et al.</u> (1991) <u>Nucleic Acid Res</u> 19:5081; Ohtsuka <u>et al.</u> (1985) <u>J Biol Chem</u> 260:2605-2608; Cassol <u>et al.</u> (1992) ; Rossolini <u>et al.</u> (1994) <u>Mol Cell Probes</u> 8:91-98). The term nucleic acid is used
25   interchangeably with gene, cDNA, and mRNA encoded by a gene.

"Nucleic acid derived from a gene" refers to a nucleic acid for whose synthesis the gene, or a subsequence thereof, has ultimately served as a template. Thus, an mRNA, a cDNA reverse transcribed from an mRNA, an RNA transcribed from that cDNA, a DNA amplified from the cDNA, an RNA transcribed from the amplified DNA, *etc.*, are all
30   derived from the gene and detection of such derived products is indicative of the presence and/or abundance of the original gene and/or gene transcript in a sample.

A nucleic acid is "operably linked" with another nucleic acid sequence when it is placed into a functional relationship with another nucleic acid sequence. For instance, a

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promoter or enhancer is operably linked to a coding sequence if it increases the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from

5   the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

The term "cytokine" includes, for example, interleukins, interferons, chemokines, hematopoietic growth factors, tumor necrosis factors and transforming growth factors. In general these are small molecular weight proteins that regulate maturation,

10   activation, proliferation, and differentiation of cells of the immune system.

A "variant" of a polypeptide is a polypeptide that differs in one or more amino acid residues from a parent or reference polypeptide, usually in at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acid or more residues.

A "variant" of a nucleic acid is a nucleic acid that differs in one or more

15   nucleic acid residues from a parent or reference nucleic acid, usually in at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 21, 24, 27, 30, 33, 36, 39, 40, 45, 50 or more nucleic acid residues.

Various additional terms are defined or otherwise characterized herein.

POLYNUCLEOTIDES OF THE INVENTION

20   NCSM Polynucleotide Sequences
The invention provides isolated or recombinant NCSM polypeptides and fragments thereof, and isolated or recombinant polynucleotides encoding said polypeptides and fragments thereof. The term "NCSM polynucleotide" is intended throughout to include nucleic acid fragments, homologues, and variants of the polynucleotide sequences

25   specifically disclosed herein unless otherwise noted.

In one aspect, the polynucleotides and polypeptides of the invention were made in two rounds of recursive sequence recombination using DNA recombination methods and formats described below. In preparation, prior to the rounds, cDNAs encoding, e.g., primate (rhesus monkey, baboon, and orangutan), cow, cat, and rabbit, B7-1

30   related sequences were cloned from their respective species, either from cell lines or peripheral blood. The cDNAs of the invention encoding baboon B7-1 and orangutan B7-1 are examples of previously unknown WT B7-1 polynucleotides. Baboon and orangutan B7-

1 have CD28 and CTLA-4 binding properties and T cell proliferation properties similar to those of hB7-1 (data not shown). The polynucleotide sequences encoding baboon (SEQ ID NO:46) and orangutan (SEQ ID NO:47) B7-1, corresponding baboon B7-1 (SEQ ID NO:93) and orangutan (SEQ ID NO:94) B7-1 polypeptides, and homologues, fragments

5   (e.g., ECD), fusion proteins thereof, are aspects of the invention.

In Round 1, the cDNAs encoding human, primate, cow, cat, and rabbit B7-1 were recursively recombined to form libraries comprising two or more recombinant polynucleotides. Other methods for obtaining libraries of recombinant polynucleotides (including NCSM polynucleotides) and/or for obtaining diversity in nucleic acids used as

10   the substrates for recursive sequence recombination are also described *infra*. The libraries of Round 1 were initially screened via three methods. An initial screening sorted the pooled recombined clones based on preferential binding ability to soluble CD28 and CTLA-4 receptor fusion proteins. A second screening selected individual clones based on the ability to bind to either CD28 or CTLA-4. A third screening tested the individual clones from the

15   second screen based on the ability to induce or inhibit T cell proliferation in conjunction with costimulation of T cell receptor/CD3 (by, e.g., an antigen or anti-CD3 Ab). Exemplary NCSM nucleic acids from Round 1 encoding NCSM polypeptides having a preferential or similar binding to CD28 relative to CTLA-4, designated as CD28 binding proteins ("CD28BP"), as compared to the binding of WT hB7-1 to CD28 relative to CTLA-4, and/or

20   having an ability to induce proliferation of T cells with T cell receptor co-engagement (e.g., in conjunction with stimulation of T cell receptor by, e.g., an antigen or anti-CD3 Ab) are shown in SEQ ID NOS:1-4, which encode NCSM polypeptides identified herein as SEQ ID NOS:48-51. Exemplary NCSM nucleic acids from Round 1 encoding NCSM polypeptides having a preferential or similar binding to CTLA-4 relative to CD28, designated as CTLA-4

25   binding proteins ("CTLA-4BP"), as compared to the binding of WT hB7-1 to CTLA-4 relative to CD28, and/or having an ability to inhibit proliferation of T cells with T cell receptor co-engagement (e.g., in conjunction with stimulation of T cell receptor by, e.g., an antigen or anti-CD3 mAb) are shown in SEQ ID NOS:22-26, which encode NCSM polypeptides identified herein as SEQ ID NOS:69-73.

30   Exemplary clones from Round 1 were further recombined in Round 2 to form recombinant polynucleotide libraries. Similar screenings were done as in Round 1 for the polynucleotide clones produced in Round 2. Exemplary recursively recombined NCSM nucleic acids encoding NCSM polypeptides having a preferential or similar binding to

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CD28 relative to CTLA-4 as compared to the binding of WT hB7-1 to CD28 relative to CTLA-4 (e.g., CD28BP polypeptides), and/or having an ability to induce proliferation of T cells in conjunction with stimulation of T cell receptor in SEQ ID NOS:5-21 and SEQ ID NOS:95-142, which encode NCSM polypeptides identified herein as SEQ ID NOS:52-68,

5   SEQ ID NOS:174-221. Additional identified recombinant CD28BP polypeptides that were identified include SEQ ID NOS:283-285 and 289-293.

Exemplary nucleic acids from Round 2 encoding NCSM polypeptides having preferential binding to CTLA-4 relative to CD28 as compared to the binding of WT hB7-1 to CTLA-4 relative to CD28 (e.g., CTLA-4BP polypeptides), and/or having an

10 ability to inhibit proliferation of T cells in conjunction with stimulation of T cell receptor are described in SEQ ID NOS:27-45 and SEQ ID NOS:143-262, which encode NCSM polypeptides identified herein as SEQ ID NOS:74-92 and SEQ ID NOS:222-252, and 263-272. Additional recombinant CTLA-4BP polypeptides that were identified include SEQ ID NOS: 286-288.

15   The term "preferential binding" in reference to an ability of an NCSM polypeptide of the invention to bind or specifically bind a CD28 receptor and/or CTLA-4 receptor typically refers to a preferential ability of the NCSM polypeptide to bind one or both of these two receptors (or to bind one such receptor relative to the other) as compared to the ability of a WT B7-1 (e.g., human, primate, or mammalian B7-1) to bind one or both

20 of these two receptors (or to bind one such receptor relative to the other). A ligand's preferential binding to a receptor typically refers to a greater, enhanced, or improved binding of the ligand to the receptor as compared to the binding of a control molecule to the receptor.

The ratio of the relative binding affinity of each CD28BP polypeptide for

25 CD28 and CTLA-4 was determined and defined as the CD28/CTLA-4 binding affinity ratio. A ratio of the relative binding affinity of WT hB7-1 for CD28 and CTLA-4 was also determined for comparison. The CD28/CTLA-4 binding affinity ratios of the CD28BP polypeptides were each found to be at least about equal to or greater than the CD28/CTLA-4 binding affinity ratio of WT hB7-1. The ratio of the relative binding affinity of each

30 CTLA-4BP polypeptide for CD28 and CTLA-4 was also determined (i.e., CTLA-4/CD28 binding affinity ratio). A ratio of the relative binding affinity of WT hB7-1 for each of CTLA-4 and CD28 was also determined. The CTLA-4/CD28 binding affinity ratios of the

CTLA-4BP polypeptides were each found to be at least about equal to or greater than the CTLA-4/CD28 binding affinity ratio of WT hB7-1.

Assays for detecting the production of specific cytokines were also performed, as described in detail below, to identify NCSM polynucleotides of the invention encoding NCSM polypeptides of the invention.

The cloned WT cow B7-1 (SEQ ID NO:280) and WT rabbit B7-1 (SEQ ID NO:281) polypeptides also worked in the binding and T cell functional assays described herein. Both cow and rabbit B7-1 polypeptides induced a T cell response in human T cells. The relative CD28/CTLA-4 binding affinity ratio of WT cow B7-1 polypeptide was found to be significantly greater than that of WT hB7-1. The relative CD28/CTLA-4 binding affinity ratio of WT rabbit B7-1 polypeptide was found to be greater than that of WT hB7-1 (data not shown).

While NCSM polynucleotides of the present invention were identified principally using the cell-based proliferation assays, receptor binding assays, and cytokine production assays described *supra* and *infra*, other assays that rely on alternative means of detection are equally suitable. For example, induction of other visual markers by receptor binding can be favorably employed. Similarly, direct binding to a receptor, e.g., by Biacore plasmon resonance, can be utilized. Other specific cytokine assays well known in the art and used for analysis of B7-1 molecules can also be utilized.

Soluble NCSM peptide constructs, including, e.g., extracellular domains of NCSM polypeptides, or fragments or subsequences thereof, alone or fused to immunoglobulin (Ig) polypeptide sequences, were also made and analyzed for receptor binding, ability to enhance or reduce an immune response, e.g., inhibit or augment T cell proliferation and/or activation, and ability to produce specific cytokine or alter or augment their levels. Nucleotide coding sequences for these soluble NCSM peptides constructs were determined. As described in detail below, such soluble NCSM polypeptides are useful in a variety of therapeutic, prophylactic, and/or diagnostic applications and methods.

The NCSM polynucleotides of the present invention that encode NCSM polypeptides are useful in a variety of applications discussed in greater detail below. For example, NCSM polynucleotides can be incorporated into expression vectors useful for gene therapy, DNA vaccination, and immunotherapy. Such vectors comprising NCSM polynucleotides encoding NCSM polypeptides are useful in clinical and medical applications in which it is desirable to provide specific proliferation/activation or anti-

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proliferation/inactivation of T cells that have encountered their specific antigen. Such vectors comprising NCSM polynucleotides of the invention are also useful in applications designed to break or avoid tolerance (e.g., vaccine or T cell adjuvants, treatment of malignant diseases and treatment of chronic infectious diseases), as where an enhanced

5   immune response is desirable, or applications designed to induce tolerance (e.g., autoimmunity, severe allergy/asthma and organ transplantation), as where a decreased immune response is desirable.

CD28BP Polynucleotides

The invention provides nucleic acids that encode NCSM polypeptides and

10   fragments thereof, wherein such polypeptides and fragments thereof bind either or both of CD28 or CTLA-4 receptor and/or modulates a T cell response. The binding of molecules can generally be considered specific if the binding affinity is about $1 \times 10^2 \, M^{-1}$ to about $1 \times 10^7 \, M^{-1}$ (i.e., about 10^{-2} - about 10^{-7} M) or greater. A "CD28 binding protein" or "CD28 binding polypeptide" ("CD28BP") refers generally to a protein or polypeptide, or fragment

15   or subsequence thereof (such as, e.g., an ECD or trunECD), that binds to or associates with a CD28 receptor. A "CTLA-4 binding protein" or "CTLA-4 binding polypeptide" ("CTLA-4BP") refers generally to a protein or polypeptide, or fragment or subsequence thereof (such as, e.g., an ECD or trunECD), that binds to or associates with a CTLA-4 receptor. A CD28BP polynucleotide is a nucleic acid sequence that encodes a CD28BP amino acid

20   sequence. A CTLA-4BP polynucleotide is a nucleic acid sequence that encodes a CTLA-4BP amino acid sequence. Some such CD28BP polypeptides (or nucleic acid encoding them) exhibit an ability to induce a T cell proliferation or activation response about equal to or greater than that of hB7-1. Some such CTLA-4BPs (or nucleic acids encoding them) exhibit an ability to induce a T cell proliferation or activation response about equal to or less

25   than that of hB7-1.

In one aspect, the invention provides isolated or recombinant nucleic acids that each comprise a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:1-21 and 95-142, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID

30   NOS:48-68, 174-221, 283-285, and 290-293, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which, but for codon degeneracy, hybridizes under at least stringent or highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b); and (d) a polynucleotide sequence comprising all or a

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fragment of (a), (b), or (c), wherein the fragment encodes a polypeptide having a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1 and/or an ability to induce a T cell proliferation or activation response about equal to or greater than that of hB7-1.

5   Also provided are isolated or recombinant nucleic acids, each comprising a nucleotide sequence selected from the group of: (a) a nucleotide sequence that encodes an extracellular domain (ECD), said nucleotide sequence comprising an ECD coding subsequence of a polynucleotide sequence selected from the group of SEQ ID NOS:1-21 and 95-142, or a complementary nucleotide sequence thereof; (b) a nucleotide sequence

10   encoding an ECD, said ECD comprising an amino acid subsequence of a polypeptide sequence selected from the group of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary nucleotide sequence thereof; and (c) a nucleotide sequence that, but for the degeneracy of the genetic code, hybridizes under at least stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b), wherein said nucleotide

15   sequence encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1 and/or an ability to induce a T cell proliferation response equal to or greater than that induced by human B7-1. Typically, the nucleotide sequence of (c) hybridizes under at least stringent conditions over substantially the entire length of polynucleotide sequence (a) and encodes a polypeptide that has a CD28/CTLA-4 binding

20   affinity ratio greater than that of hB7-1.

Some such nucleic acids further comprise at least a second nucleotide sequence that encodes a signal peptide, said second nucleotide sequence selected from the group of: (a) a nucleotide sequence comprising a signal peptide coding subsequence of a polynucleotide sequence selected from the group of SEQ ID NOS:1-21 and 95-142, or a

25   complementary nucleotide sequence thereof; (b) a nucleotide sequence encoding a signal peptide, which comprises an amino acid subsequence of a polypeptide sequence selected from the group of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary nucleotide sequence thereof; (c) a nucleotide sequence that, but for the degeneracy of the genetic code, hybridizes under at least stringent conditions over

30   substantially the entire length of polynucleotide sequence (a) or (b), wherein said nucleotide sequence encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1; and (d) a nucleotide sequence encoding a signal peptide of a B7-1 polypeptide. In some aspects, the nucleotide sequence of (c) hybridizes under at

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least stringent conditions over substantially the entire length of polynucleotide sequence (a) and encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio greater than that of hB7-1 and/or an ability to induce a T cell proliferation response about equal to or greater than that induced by human B7-1.

5   Some such nucleic acids further comprising at least a third nucleotide sequence encoding a transmembrane domain selected from the group of: (a) a nucleotide sequence comprising a TMD coding subsequence of a polynucleotide sequence selected from the group of SEQ ID NOS:1-21 and 95-142, or a complementary nucleotide sequence thereof; (b) a nucleotide sequence encoding a TMD, which comprises an amino acid

10   subsequence of a polypeptide sequence selected from the group of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary nucleotide sequence thereof; (c) a nucleotide sequence that, but for the degeneracy of the genetic code, hybridizes under at least stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b), wherein said nucleotide sequence encodes a polypeptide that has a CD28/CTLA-4

15   binding affinity ratio about equal to or greater than that of hB7-1; and (d) a nucleotide sequence that encodes a TMD of a B7-1 polypeptide. Some polypeptides further comprise at least a fourth nucleotide sequence encoding a cytoplasmic domain selected from the group of: (a) a nucleotide sequence comprising a CD coding subsequence of a polynucleotide sequence selected from the group of SEQ ID NOS:1-21 and 95-142, or a

20   complementary nucleotide sequence thereof; (b) a nucleotide sequence encoding a CD, which CD comprises an amino acid subsequence of a polypeptide sequence selected from the group of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary nucleotide sequence thereof; (c) a nucleotide sequence that, but for the degeneracy of the genetic code, hybridizes under at least stringent conditions over substantially the entire

25   length of polynucleotide sequence (a) or (b), wherein said nucleotide sequence encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1; and (d) a nucleotide sequence that encodes a CD of a B7-1 polypeptide.

In another aspect, the invention provides isolated or recombinant nucleic acids that each comprises a polynucleotide sequence encoding a polypeptide, wherein the

30   encoded polypeptide comprises an amino acid sequence which is (a) substantially identical over at least about 100 contiguous amino acid residues of any one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293 and (b) is a non naturally-occurring sequence. In some instances, the encoded polypeptide is substantially identical over at least about 150 or about

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200 contiguous amino acid residues of any one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293.

In yet another aspect, the invention provides isolated or recombinant nucleic acids that each comprise a nucleotide sequence coding for a polypeptide comprising the amino acid sequence set forth in any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a subsequence thereof, wherein the subsequence comprises at least one of the signal sequence, ECD, transmembrane domain, and cytoplasmic domain of the polypeptide, and wherein the amino acid sequence or subsequence is a non naturally-occurring sequence.

For some such isolated or recombinant polypeptides, the polypeptide comprises an ECD amino acid sequence encoded by an ECD coding nucleotide sequence, and the ECD coding nucleotide sequence comprises a nucleotide sequence that, but for codon degeneracy, hybridizes under at least stringent conditions over substantially the entire length of the ECD coding nucleotide sequence of a polynucleotide sequence selected from any of SEQ ID NOS:1-21 and 95-142 or the nucleotide coding sequence that encodes the ECD of a polypeptide selected from any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293. Some such isolated or recombinant polypeptides further comprises a signal peptide amino acid sequence encoded by a signal peptide coding nucleotide sequence. The signal peptide coding nucleotide sequence selected from the group of: (a) a nucleotide sequence of a polynucleotide sequence selected from any of SEQ ID NOS:1-21 and 95-142, wherein said nucleotide sequence encodes a signal peptide; (b) a nucleotide sequence that encodes the signal peptide of a polypeptide selected from any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293; and (c) a nucleotide sequence which, but for codon degeneracy, hybridizes under at least stringent conditions over substantially the entire length of a nucleotide sequence (a) or (b).

Some such isolated or recombinant polypeptides further comprise a transmembrane domain (TMD) amino acid sequence encoded by a TMD nucleotide sequence selected from the group of: (a) a nucleotide sequence of a polynucleotide sequence selected from any of SEQ ID NOS:1-21 and 95-142, wherein said nucleotide sequence encodes a TMD polypeptide; (b) a nucleotide sequence that encodes the TMD of a polypeptide selected from any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293; and (c) a nucleotide sequence which, but for codon degeneracy, hybridizes under at least stringent or highly stringent conditions over substantially the entire length of a nucleotide sequence (a) or (b).

Some such isolated or recombinant polypeptides further comprise a cytoplasmic domain (CD) amino acid sequence encoded by a CD nucleotide sequence selected from the group of: (a) a nucleotide sequence of a polynucleotide sequence selected from any of SEQ ID NOS:1-21 and 95-142, wherein said nucleotide sequence encodes a CD

5   polypeptide; (b) a nucleotide sequence that encodes the CD of a polypeptide selected from any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293; and (c) a nucleotide sequence which , but for codon degeneracy, hybridizes under at least stringent or highly stringent conditions over substantially the entire length of a nucleotide sequence (a) or (b).

For some of the CD28BP nucleic acids described above, the polypeptide

10   encoded by the nucleic acid has one of more of the following properties: 1) a CD28/CTLA-4 binding affinity ratio equal to, about equal to, or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1; 2) either an equal or an enhanced binding affinity for CD28 as compared to a binding affinity of a wild type co-stimulatory molecule for CD28; 3) a decreased or a lowered binding affinity for CTLA-4 as compared to a binding affinity of a

15   wild type co-stimulatory molecule for CTLA-4; induces T-cell proliferation or T-cell activation or both; or 4) modulates T-cell activation, but does not induce proliferation of purified T-cells activated by soluble anti-CD3 mAbs.

In another embodiment, the invention provides isolated or recombinant nucleic acids each comprising a nucleotide sequence selected from the group of: (a) a

20   nucleotide sequence that encodes an extracellular domain (ECD), said nucleotide sequence comprising an ECD coding subsequence of a polynucleotide sequence selected from the group of SEQ ID NOS:1-21 and 95-142, or a complementary nucleotide sequence thereof; (b) a nucleotide sequence encoding an ECD, said ECD comprising an amino acid subsequence of a polypeptide sequence selected from the group of SEQ ID NOS:48-68,

25   174-221, 283-285, and 290-293, or a complementary nucleotide sequence thereof; and (c) a nucleotide sequence that, but for codon degeneracy, hybridizes under at least stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b), wherein said nucleotide sequence encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1

30   or an ability to induce a T cell proliferation response about equal to or greater than that induced by hB7-1.

For some such isolated or recombinant nucleic acids, the nucleotide sequence of (c) hybridizes under at least stringent conditions over substantially the entire length of

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polynucleotide sequence (a) and encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio greater than the CD28/CTLA-4 binding affinity ratio of human B7-1 or induces a T cell proliferation response greater than that induced by hB7-1.. Some such isolated or recombinant nucleic acids further comprise at least a second nucleotide sequence

5   that encodes a signal peptide, wherein said second nucleotide sequence is selected from the group of: (a) a nucleotide sequence comprising a signal peptide coding subsequence of a polynucleotide sequence selected from the group of SEQ ID NOS:1-21 and 95-142, or a complementary nucleotide sequence thereof; (b) a nucleotide sequence encoding a signal peptide, said signal peptide comprising an amino acid subsequence of a polypeptide

10   sequence selected from the group of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary nucleotide sequence thereof; (c) a nucleotide sequence that, but for codon degeneracy, hybridizes under at least stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b), wherein said nucleotide sequence encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than

15   that of hB7-1, or an ability to induce a T cell proliferation or activation response about equal to or greater than that induced by hB7-1; and (d) a nucleotide sequence encoding a signal peptide of a B7-1 polypeptide.

For some such isolated or recombinant nucleic acids, the nucleotide sequence of (c) hybridizes, but for codon degeneracy, under at least stringent conditions over

20   substantially the entire length of polynucleotide sequence (a) and encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio greater than that of hB7-1 or an ability to induce a T cell proliferation response about equal to or greater than that induced by hB7-1. Some such nucleic acids further comprise at least a third nucleotide sequence encoding a transmembrane domain selected from the group of: (a) a nucleotide sequence comprising a

25   transmembrane domain coding subsequence of a polynucleotide sequence selected from the group of SEQ ID NOS:1-21 and 95-142, or a complementary nucleotide sequence thereof; (b) a nucleotide sequence encoding a transmembrane domain, said transmembrane domain comprising an amino acid subsequence of a polypeptide sequence selected from the group of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary nucleotide

30   sequence thereof; (c) a nucleotide sequence that hybridizes, but for the degeneracy of the genetic code, under at least stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b), wherein said nucleotide sequence encodes a polypeptide

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that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1; and (d) a nucleotide sequence that encodes a transmembrane domain of a B7-1 polypeptide.

Further, some such nucleic acids further comprise at least a fourth nucleotide sequence encoding a cytoplasmic domain selected from the group of: (a) a nucleotide

5 sequence comprising a cytoplasmic domain coding subsequence of a polynucleotide sequence selected from the group of SEQ ID NOS:1-21 and 95-142, or a complementary nucleotide sequence thereof; (b) a nucleotide sequence encoding a cytoplasmic domain, said cytoplasmic domain comprising an amino acid subsequence of a polypeptide sequence selected from the group of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a

10 complementary nucleotide sequence thereof; (c) a nucleotide sequence that, but for codon degeneracy, hybridizes under at least stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b), wherein said nucleotide sequence encodes a polypeptide that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than that ratio of human B7-1; and (d) a nucleotide sequence that encodes a cytoplasmic domain

15 of a B7-1 polypeptide.

CTLA-4BP Polynucleotides

The invention includes isolated or recombinant nucleic acids that each comprise a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:22-45, 143-173, or a complementary polynucleotide sequence thereof;

20 (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:69-92, 222-247, 286-289, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which, but for codon degeneracy, hybridizes under at least stringent or highly stringent conditions over substantially the entire full-length length of polynucleotide sequence (a) or (b); and (d) a polynucleotide sequence comprising all or a

25 fragment of (a), (b), or (c); wherein (c) or (d) encodes a polypeptide having a naturally-occurring or non naturally-occurring sequence comprising at least one of: Gly at position 2; Thr at position 4; Arg at position 5; Gly at position 8; Pro at position 12; Met at position 25; Cys at position 27; Pro at position 29; Leu at position 31; Arg at position 40; Leu at position 52; His at position 65; Ser at position 78; Asp at position 80; Tyr at position 87; Lys at

30 position 120; Asp at position 122; Lys at position 129; Met at position 135; Phe at position 150; Ile at position 160; Ala at position 164; His at position 172; Phe at position 174; Leu at position 176; Asn at position 178; Asn at position 186; Glu at position 194; Gly at position 196; Thr at position 199; Ala at position 210; His at position 212; Arg at position 219; Pro

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at position 234; Asn at position 241; Leu at position 244; Thr at position 250; Ala at position 254; Tyr at position 265; Arg at position 266; Glu at position 273; Lys at position 275; Ser at position 276; an amino acid deletion at position 276; and Thr at position 279, wherein the position number corresponds to that of the human B7-1 amino acid sequence (SEQ ID NO:278), and wherein said polypeptide has a CTLA-4/CD28 binding affinity ratio about equal to, equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1.

In another aspect, the invention provides isolated or recombinant nucleic acids that comprise a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:253-262, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:263-272, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which, but for codon degeneracy, hybridizes under highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b) and encodes a polypeptide having a non naturally-occurring sequence; and (d) a polynucleotide sequence comprising all or a fragment of (a), (b), or (c), wherein the fragment encodes a polypeptide having (i) a naturally-occurring or non naturally-occurring sequence and (ii) a CTLA-4/CD28 binding affinity ratio about equal to or greater than that of human B7-1.

In another aspect, the invention provides isolated or recombinant nucleic acids comprising a polynucleotide sequence encoding a polypeptide, the encoded polypeptide comprising an amino acid sequence which is substantially identical over at least about 125, 150, 175, 200, 225, 250, or more contiguous amino acid residues of any one of SEQ ID NOS:69-92, 222-247, 263-272, and 286-289.

The invention also provides isolated or recombinant nucleic acids that each comprise a nucleotide sequence coding for a polypeptide comprising the amino acid sequence set forth in any of SEQ ID NOS:69-92, 222-247, 263-272, and 286-289, or a subsequence thereof, wherein the subsequence comprises at least one of: the signal sequence, extracellular domain, transmembrane domain, and cytoplasmic domain of said polypeptide, and wherein the amino acid sequence or subsequence is a non naturally-occurring sequence.

For some such CTLA-4BP nucleic acids described above, a polypeptide encoded therefrom has a CTLA-4/CD28 binding affinity ratio about equal to, equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1. Furthermore, the

polypeptide encoded by some such CTLA-4BP nucleic acids has either a same binding affinity or an enhanced binding affinity for CD28 as compared to a binding affinity of a wild type co-stimulatory molecule for CD28. Some such encoded polypeptides have a decreased or a lowered binding affinity for CTLA-4 as compared to a binding affinity of a wild type co-stimulatory molecule for CTLA-4 (e.g., a mammalian B7-1, such as hB7-1). Some such encoded polypeptides inhibit either or both T-cell proliferation or T-cell activation. Some such encoded polypeptides modulate T-cell activation, but do not induce proliferation of purified T-cells activated by soluble anti-CD3 mAbs.

In addition, the invention provides novel isolated or recombinant nucleic acids corresponding to baboon and orangutan B7-1. Such sequences comprise a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NO:46, SEQ ID NO:47, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NO:93, SEQ ID NO:94, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence encoding a subsequence of a polypeptide selected from SEQ ID NO:93, SEQ ID NO:94, or a complementary polynucleotide sequence thereof, wherein the subsequence comprises at least one of: the signal sequence, extracellular domain, transmembrane domain, and the cytoplasmic domain of said polypeptide. Included are polypeptide fragments (e.g., 100, 150, 200, or 250 amino acids) and nucleotides encoding such fragments, having CD28 and/or CTLA-4 binding properties similar or about equal to those of hB7-1 and/or an ability to induce T cell proliferation or activation response similar or about equal to that of hB7-1.

Additional Aspects

The invention also includes RNA sequences that correspond to each of the NCSM DNA sequences of the invention. For example, included is an RNA sequence comprising the NCSM DNA sequence of any of SEQ ID NOS:1-47, 95-173, and 253-262, wherein a uracil residue is substituted for each thymidine residue in said DNA sequence, and a complementary sequence of each such RNA sequence. Also included is a RNA sequence comprising a nucleotide sequence comprising at least one of an ECD coding sequence, TMD coding sequence, CD coding nucleotide sequence, and/or signal peptide coding nucleotide sequence of DNA sequence of any of SEQ ID NOS:1-47, 95-173, and 253-262, wherein a uracil residue is substituted for each thymidine residue in said DNA sequence, and complementary sequences of such RNA sequence. The DNA subsequence of SEQ ID NOS:1-47, 95-173, and 253-262, that encodes each of the ECD, TMD, CD, and

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signal peptide are readily determined by alignment with the DNA sequence of hB7-1 (see, e.g., Figs. 2 and 3). The invention further provides a virus comprising a nucleic acid or polynucleotide (RNA or DNA) of the invention.

Any of the CD28BP and CTLA-4BP nucleic acids described above may acid encode a fusion protein comprising at least one additional amino acid sequence. T he at least one additional amino acid sequence comprises an Ig polypeptide. The polypeptide may comprise a human IgG polypeptide or Fc domain of an IgG polypeptide, and may comprise an Fc hinge, a CH2 domain, and a CH3 domain. Exemplary IgG1 polypeptides and their sequences are shown in the Examples below.

A polypeptide encoded by any of the CD28BP and CTLA-4BP nucleic acids described above may comprise at least one of a signal sequence, a precursor peptide, and an epitope tag sequence or Histidine tag.

In another aspect, the invention provides cells comprising one or more of the CD28BP or CTLA-4BP nucleic acids described above. Such cells may express one or more polypeptides encoded by the nucleic acids of the invention.

The invention also provides vectors comprising any of the CTLA-4BP or CD28BP nucleic acids described above. Such vectors may comprise a plasmid, a cosmid, a phage, a virus, or a fragment of a virus. Such vectors may comprise an expression vector, and, if desired, the CD28BP or CTLA-4BP nucleic acid is operably linked to a promoter, including those discussed herein and below.

Such a vector may be a bicistronic vector, comprising in addition to a nucleotide sequence encoding a CD28BP or CTLA-4BP, a nucleotide sequence encoding a transgene, such as an antigen, marker, or other co-stimulatory molecule, or cytokine (e.g., GM-CSF, IL-12, or IL-2). Such vector may also be tricistronic or of higher order, comprising a further (third) nucleotide acid sequence. For example, the vector may comprise nucleotide sequences encoding an NCSM polypeptide (e.g., CD28BP or CTLA-4BP), antigen, and cytokine, such as IL-12. In one embodiment, the antigen is a cancer antigen, such as EpCam (or mutant or variant polypeptide thereof) or another cancer antigen described below, or viral antigen. In such expression vector, the nucleic acid may be operably linked to first promoter and the polynucleotide sequence encoding the antigen may operably linked to a second promoter. Each promoter can comprise any promoter described below. In one aspect, one or both promoters in the expression vector that includes a CD28BP or CTLA-4BP polypeptide-encoding nucleotide sequence is a CMV promoter or

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variant thereof. The vector may further comprise a bovine growth hormone (BGH) poly adenylation sequence or SV40 polyA sequence.

A preferred "backbone" expression vector is that shown in Figure 21; the expression vector components shown in this backbone vector may be used with any NCSM nucleic acid sequence. Other expression vector elements that can be employed and other vector types and formats are described in detail below. A preferred expression vector that includes a CD28BP or CTLA-4BP polypeptide-encoding nucleotide sequence is shown in Figure 22A. The components of a preferred bicistronic expression vector that includes a CD28BP polypeptide-encoding nucleotide sequence, such as that encoding clone CD28BP-15, and a nucleic acid sequence encoding EpCam are shown in Fig. 23A.

The invention also provides host cells comprising any of the vectors that comprise nucleotide sequences encoding any CD28BP or CTLA-4BP described herein.

Furthermore, in another aspect, the invention provides compositions comprising an excipient or carrier and at least one of any of the CD28BP or CTLA-4BP nucleic acids, or vectors, cells, or host comprising such nucleic acids. Such composition may be pharmaceutical compositions, and the excipient or carrier may be a pharmaceutically acceptable excipient or carrier.

The invention also includes compositions comprising two or more NCSM polynucleotides of the invention or fragments thereof (e.g., as substrates for recombination). The composition can comprise a library of recombinant nucleic acids, where the library contains at least 2, at least 3, at least 5, at least 10, at least 20, at least 50, or at least 100 or more nucleic acids described above. The nucleic acids are optionally cloned into expression vectors, providing expression libraries.

The NCSM polynucleotides of the invention and fragments thereof, as well as vectors comprising such polynucleotides, may be employed for therapeutic or prophylactic uses in combination with a suitable carrier, such as a pharmaceutical carrier. Such compositions comprise a therapeutically and/or prophylactically effective amount of the compound, and a pharmaceutically acceptable carrier or excipient. Such a carrier or excipient includes, but is not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration. Methods of administering nucleic acids, polypeptides, and proteins are well known in the art, and are further discussed below.

The invention also includes compositions produced by digesting one or more of any of the NCSM nucleic acids described above with a restriction endonuclease, an RNAse, or a DNAse (e.g., as is performed in certain of the recombination formats noted above); and compositions produced by fragmenting or shearing one or more NCSM

5   polynucleotides of the invention by mechanical means (e.g., sonication, vortexing, and the like), which can also be used to provide substrates for recombination in the methods described herein. The invention also provides compositions produced by cleaving at least one of any of the CD28BP or CTLA-4BP nucleic acids described above. The cleaving may comprise mechanical, chemical, or enzymatic cleavage, and the enzymatic cleavage may

10   comprise cleavage with a restriction endonuclease, an RNAse, or a DNAse.

Also included in the invention are compositions produced by a process comprising incubating one or more of the fragmented nucleic acid sets in the presence of ribonucleotide or deoxyribonucleotide triphosphates and a nucleic acid polymerase. This resulting composition forms a recombination mixture for many of the recombination

15   formats noted above. The nucleic acid polymerase may be an RNA polymerase, a DNA polymerase, or an RNA-directed DNA polymerase (e.g., a "reverse transcriptase"); the polymerase can be, e.g., a thermostable DNA polymerase (e.g., VENT, TAQ, or the like).

Similarly, compositions comprising sets of oligonucleotides corresponding to more than one NCSM nucleic acids of the invention are useful as recombination substrates

20   and are a feature of the invention. For convenience, these fragmented, sheared, or oligonucleotide synthesized mixtures are referred to as fragmented nucleic acid sets.

In one aspect, the invention provides an isolated or recombinant nucleic acid encoding a polypeptide that has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of hB7-1, produced by mutating or

25   recombining at least one CTLA-4BP nucleic acid described above. In another aspect, the invention provides an isolated or recombinant nucleic acid encoding a polypeptide that has a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of hB7-1, produced by mutating or recombining at least one CD28BP nucleic acid described above.

30   The invention also provides a chimeric or recombinant polynucleotide that encodes a polypeptide having a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of hB7-1. In some aspects, such encoded polypeptide is a mammalian B7-1 variant. In some aspects, such polypeptide

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comprises an amino acid sequence comprising one or more amino acid subsequences corresponding to amino acid subsequences of wild-type cow B7-1, baboon B7-1, rabbit B7-1, and human B7-1 polypeptides. In some aspects, such polypeptide exhibits an ability to induce a T cell proliferation or activation response of T cells (e.g., stimulated by anti-CD3

5   Abs or antigen) greater than that of cow B7-1, rabbit B7-1 or human B7-1. Chimeric or recombinant polypeptides encoded therefrom are also an aspect of the invention (see, e.g., Figure 8B).

In addition, the invention includes a chimeric or recombinant polynucleotide that encodes a polypeptide having a CTLA-4/CD28 binding affinity ratio about equal to or

10   greater than that of hB7-1. In some aspects, such encoded polypeptide is a mammalian B7-1 variant. In some aspects, such polypeptide comprises an amino acid sequence comprising one or more amino acid subsequences corresponding to amino acid subsequences of wild-type rhesus B7-1, baboon B7-1, human B7-1, orangutan B7-1, and cow B7-1 polypeptides. In some aspects, such polypeptide exhibits an ability to suppress or inhibit a T cell

15   proliferation or activation response (e.g., of T cells stimulated by anti-CD3 Abs or antigen) relative to that induced human B7-1. Chimeric or recombinant polypeptides encoded therefrom are also an aspect of the invention (see, e.g., Figure 8A).

Making Polynucleotides

NCSM polynucleotides, oligonucleotides, and nucleic acid fragments of the

20   invention can be prepared by standard solid-phase methods, according to known synthetic methods. Typically, fragments of up to about 100 bases are individually synthesized, then joined (e.g., by enzymatic or chemical ligation methods, or polymerase mediated recombination methods) to form essentially any desired continuous sequence. For example, the NCSM polynucleotides and oligonucleotides of the invention can be prepared by

25   chemical synthesis using, e.g., classical phosphoramidite method described by, e.g., Beaucage et al. (1981) Tetrahedron Letters 22:1859-69, or the method described by Matthes et al. (1984) EMBO J 3:801-05, e.g., as is typically practiced in automated synthetic methods. According to the phosphoramidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned into appropriate

30   vectors.

In addition, essentially any nucleic acid can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (mcrc@oligos.com), The Great American Gene Company (http://www.genco.com),

ExpressGen Inc. (www.expressgen.com), Operon Technologies Inc. (Alameda, CA) and many others. Similarly, peptides and antibodies can be custom ordered from any of a variety of sources, e.g., PeptidoGenic (pkim@ccnet.com), HTI Bio-products, Inc. (http://www.htibio.com), BMA Biomedicals Ltd. (U.K.), Bio.Synthesis, Inc., and many others.

5   Certain NCSM polynucleotides of the invention may also be obtained by screening cDNA libraries (e.g., libraries generated by recombining homologous nucleic acids as in typical recursive sequence recombination methods) using oligonucleotide probes that can hybridize to or PCR-amplify polynucleotides which encode the NCSM polypeptides and fragments of those polypeptides. Procedures for screening and isolating
10   cDNA clones are well-known to those of skill in the art. Such techniques are described in, e.g., Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymol. Vol. 152, Acad. Press, Inc., San Diego, CA ("Berger"); Sambrook, *supra*, and Current Protocols in Molecular Biology, Ausubel, *supra*. Some NCSM polynucleotides of the invention can be obtained by altering a naturally occurring backbone, e.g., by mutagenesis,
15   recursive sequence recombination (e.g., shuffling), or oligonucleotide recombination. In other cases, such polynucleotides can be made in silico or through oligonucleotide recombination methods as described in the references cited herein.

        As described in more detail herein, the NCSM polynucleotides of the invention include polynucleotide sequences that encode NCSM polypeptide sequences and
20   fragments thereof (including all forms of soluble NCSM polypeptides and fusion proteins), polynucleotide sequences complementary to these polynucleotide sequences and fragments thereof, polynucleotides that hybridize under at least stringent conditions to NCSM sequences defined herein, novel fragments of coding sequences and complementary sequences thereof, and variants, analogs, and homologue derivatives of all of the above. A
25   coding sequence refers to a nucleotide sequence encodes a particular polypeptide or domain, region, or fragment of said polypeptide. A coding sequence may code for a NCSM polypeptide or fragment thereof having a functional property, such as a an ability to bind a receptor, induce or suppress T cell proliferation in conjunction with stimulation of T cell receptor (by, e.g., an antigen or anti-CD3 Ab), or induce or stimulate a cytokine response as
30   described herein. The polynucleotides of the invention can be in the form of RNA or in the form of DNA, and include mRNA, cRNA, synthetic RNA and DNA, and cDNA. The polynucleotides can be double-stranded or single-stranded, and if single-stranded, can be the coding strand or the non-coding (anti-sense, complementary) strand. The NCSM

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polynucleotides optionally include the coding sequence of a NCSM polypeptide (i) in isolation, (ii) in combination with one or more additional coding sequences, so as to encode, e.g., a fusion protein, a pre-protein, a prepro-protein, or the like, (iii) in combination with non-coding sequences, such as introns, control elements, such as a promoter (e.g., naturally

5   occurring or recombinant or shuffled promoter), a terminator element, or 5' and/or 3' untranslated regions effective for expression of the coding sequence in a suitable host, and/or (iv) in a vector, cell, or host environment in which NCSM coding sequence is a heterologous gene. The NCSM polynucleotides include the respective coding sequences of components of a NCSM polypeptide, including, e.g., the coding sequence for each of the

10   signal peptide, ECD, transmembrane domain, cytoplasmic domain, mature region, and fragments thereof, and variants, analogs, and homologue derivatives thereof. Polynucleotide sequences can also be found in combination with typical compositional formulations of nucleic acids, including in the presence of carriers, buffers, adjuvants, excipients, and the like, as are known to those of ordinary skill in the art. NCSM nucleotide

15   fragments typically comprise at least about 500 nucleotide bases, usually at least about 600, 650, or 700 bases, and often 750 or more bases. The nucleotide fragments, variants, analogs, and homologue derivatives of NCSM polynucleotides may have hybridize under highly stringent conditions to a NCSM polynucleotide or homologue sequence described herein and/or encode amino acid sequences having at least one of the properties of receptor

20   binding, ability to alter an immune response via, e.g., T cell activation /proliferation, and cytokine production of NCSM polypeptides described herein.

Using Polynucleotides

The NCSM polynucleotides and fragments, variants, and homologues thereof of the invention have a variety of uses in, for example, recombinant production (i.e.,

25   expression) of the NCSM polypeptides of the invention typically through expression of a plasmid expression vector comprising a sequence encoding a NCSM polypeptide or fragment thereof (e.g., ECD domain); as therapeutics; as prophylactics; as diagnostic tools; as immunogens; as adjuvants; as diagnostic probes for the presence of complementary or partially complementary nucleic acids (including for detection of natural B7-1 or related co-

30   stimulatory molecule coding nucleic acids) as substrates for further reactions, e.g., recursive sequence recombination reactions or mutation reactions to produce new and/or improved homologues, and the like. Such NCSM polynucleotides and fragments, variants, and homologues thereof of the invention can be administered to a subject by any one of the

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delivery routes described below (including, but not limited to, e.g., intramuscularly, intradermally, subdermally, subcutaneously, orally, intraperitoneally, intrathecally, intravenously, mucosally, systemically, parenterally, via inhalation, or placed within a cavity of the body (including, e.g., during surgery)).

5 EXPRESSION OF POLYPEPTIDES FROM POLYNUCLEOTIDES

In accordance with the present invention, NCSM polynucleotide sequences which encode novel full-length or mature NCSM polypeptides or proteins, fragments, variants or homologues thereof, related fusion polypeptides or proteins, or functional equivalents thereof, collectively referred to herein, e.g., as "NCSM" molecules, are used in

10 recombinant DNA molecules that direct the expression of the NCSM polypeptides in appropriate host cells. Due to the inherent degeneracy of the genetic code, other nucleic acid sequences that encode substantially the same or a functionally equivalent amino acid sequence are also used to synthesize, clone and express the NCSM polypeptides.

Modified Coding Sequences

15 As will be understood by those of ordinary skill in the art, it can be advantageous to modify a coding sequence to enhance its expression in a particular host. The genetic code is redundant with 64 possible codons, but most organisms preferentially use a subset of these codons. The codons that are utilized most often in a species are called optimal codons, and those not utilized very often are classified as rare or low-usage codons

20 (see, e.g., Zhang, S. P. et al. (1991) Gene 105:61-72). Codons can be substituted to reflect the preferred codon usage of the host, a process called "codon optimization" or "controlling for species codon bias."

Optimized coding sequence containing codons preferred by a particular prokaryotic or eukaryotic host (see, e.g., Murray, E. et al. (1989) Nuc Acids Res 17:477-

25 508) can be prepared, for example, to increase the rate of translation or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, as compared with transcripts produced from a non-optimized sequence. Translation stop codons can also be modified to reflect host preference. For example, preferred stop codons for S. cerevisiae and mammals are UAA and UGA respectively. The preferred stop codon

30 for monocotyledonous plants is UGA, whereas insects and E. coli prefer to use UAA as the stop codon (Dalphin, M.E. et al. (1996) Nuc Acids Res 24:216-218).

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The polynucleotide sequences of the present invention can be engineered in order to alter an NCSM coding sequence of the invention for a variety of reasons, including but not limited to, alterations which modify the cloning, processing and/or expression of the gene product. For example, alterations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis, to insert new restriction sites, to alter glycosylation and/or pegylation patterns, to change codon preference, to introduce splice sites, etc. Further details regarding silent and conservative substitutions are provided below.

Vectors, Promoters, and Expression Systems

The present invention also includes recombinant constructs comprising one or more of the nucleic acid sequences as broadly described above. The constructs comprise a vector, such as, a plasmid, a cosmid, a phage, a virus, a virus-like particle, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), and the like, into which a nucleic acid sequence of the invention (e.g., one which encodes a NCSM polypeptide or fragment thereof) has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the nucleic acid sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available.

General texts that describe molecular biological techniques useful herein, including the use of vectors, promoters and many other relevant topics, include Berger, *supra*; Sambrook (1989), *supra*, and Ausubel, *supra*. Examples of techniques sufficient to direct persons of skill through in vitro amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (e.g., NASBA), e.g., for the production of the homologous nucleic acids of the invention are found in Berger, Sambrook, and Ausubel, all *supra*, as well as Mullis et al. (1987) U.S. Patent No. 4,683,202; PCR Protocols: A Guide to Methods and Applications (Innis et al., eds.) Academic Press Inc. San Diego, CA (1990) ("Innis"); Arnheim & Levinson (October 1, 1990) C&EN 36-47; The Journal Of NIH Research (1991) 3:81-94; (Kwoh et al. (1989) Proc Natl Acad Sci USA 86:1173-1177; Guatelli et al. (1990) Proc Natl Acad Sci USA 87:1874-1878; Lomeli et al. (1989) J Clin Chem 35:1826-1831; Landegren et al. (1988) Science 241:1077-1080; Van Brunt (1990) Biotechnology 8:291-294; Wu and Wallace (1989) Gene 4:560-569; Barringer et al. (1990) Gene 89:117-122, and Sooknanan and Malek (1995) Biotechnology 13:563-564. Improved

methods of cloning in vitro amplified nucleic acids are described in Wallace et al., U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids by PCR are summarized in Cheng et al. (1994) Nature 369:684-685 and the references therein, in which PCR amplicons of up to 40 kilobases (kb) are generated. One of skill will appreciate that

5   essentially any RNA can be converted into a double stranded DNA suitable for restriction digestion, PCR expansion and sequencing using reverse transcriptase and a polymerase. See Ausubel, Sambrook and Berger, all *supra*.

The present invention also provides host cells that are transduced with vectors of the invention, and the production of polypeptides of the invention by recombinant

10   techniques. Host cells are genetically engineered (e.g., transduced, transformed or transfected) with the vectors of this invention, which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants, or

15   amplifying the NCSM gene. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to those skilled in the art and in the references cited herein, including, e.g., Freshney (1994) Culture of Animal Cells, a Manual of Basic Technique, third edition, Wiley- Liss, New York and the references cited therein.

20   The NCSM polypeptides of the invention can also be produced in non-animal cells such as plants, yeast, fungi, bacteria and the like. In addition to Sambrook, Berger and Ausubel, details regarding cell culture are found in, e.g., Payne et al. (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc. New York, NY; Gamborg and Phillips (eds.) (1995) Plant Cell, Tissue and Organ Culture; Fundamental

25   Methods Springer Lab Manual, Springer-Verlag (Berlin Heidelberg NY); Atlas & Parks (eds.) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, FL.

The polynucleotides of the present invention and fragments and variants thereof, which encode the NCSM polypeptide molecules, may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include

30   chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40, bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, pseudorabies, adeno-associated virus, retroviruses and many others. Any vector

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that transduces genetic material into a cell, and, if replication is desired, which is replicable and viable in the relevant host can be used.

The nucleic acid sequence in the expression vector is operatively linked to an appropriate transcription control sequence (promoter) to direct mRNA synthesis. Examples
5 of such promoters include: LTR or SV40 promoter, *E. coli* lac or trp promoter, phage lambda P_L promoter, CMV promoter, and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation, and a transcription terminator. The vector optionally includes appropriate sequences for amplifying expression, e.g., an
10 enhancer. In addition, the expression vectors optionally comprise one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells, such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli.*

The vector containing the appropriate DNA sequence encoding a NCSM
15 polypeptide, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. Examples of appropriate expression hosts include: bacterial cells, such as *E. coli, Streptomyces,* and *Salmonella typhimurium*; fungal cells, such as *Saccharomyces cerevisiae, Pichia pastoris,* and *Neurospora crassa*; insect cells such as *Drosophila* and *Spodoptera frugiperda*;
20 mammalian cells such as CHO, COS, BHK, HEK 293 or Bowes melanoma; plant cells, etc. It is understood that not all cells or cell lines need to be capable of producing fully functional NCSM polypeptides or fragments thereof; for example, antigenic fragments of NCSM polypeptide may be produced in a bacterial or other expression system. The invention is not limited by the host cells employed.

25 In bacterial systems, a number of expression vectors may be selected depending upon the use intended for the NCSM polypeptide or fragment thereof. For example, when large quantities of a NCSM polypeptide or fragments thereof are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be desirable. Such vectors include, but are not limited to,
30 multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene), in which NCSM nucleotide coding sequence may be ligated into the vector in-frame with sequences for the amino-terminal Met and the subsequent 7 residues of beta-galactosidase

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so that a hybrid protein is produced; pIN vectors (Van Heeke & Schuster (1989) J Biol Chem 264:5503-5509); pET vectors (Novagen, Madison WI); and the like.

Similarly, in the yeast *Saccharomyces cerevisiae* a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase and PGH may be used for production of the NCSM polypeptides of the invention. For reviews, see Ausubel, *supra*, Berger, *supra*, and Grant et al. (1987) Methods in Enzymology 153:516-544.

In mammalian host cells, a number of expression systems, such as viral-based systems, may be utilized. In cases where an adenovirus is used as an expression vector, a coding sequence is optionally ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential E1 or E3 region of the viral genome results in a viable virus capable of expressing NCSM molecule in infected host cells (Logan and Shenk (1984) Proc Natl Acad Sci USA 81:3655-3659). In addition, transcription enhancers, such as the rous sarcoma virus (RSV) enhancer, are used to increase expression in mammalian host cells. Host cells, media, expression systems, and methods of production include those known for cloning and expression of various mammalian B7-1s (e.g., hB7-1 and mouse B7-1).

Promoters for use with NCSM polynucleotide sequences of the present invention include recombinant, mutated, or recursively recombined (e.g., shuffled) promoters, including optimized recombinant CMV promoters, as described in copending, commonly assigned PCT Application Serial No. US01/20123, entitled "Novel Chimeric Promoters," filed June 21, 2001 as LJAQ Attorney Docket No. 02-031910PC, incorporated herein by reference in its entirety for all purposes. Such promoters can be employed in expression vectors comprising nucleotide sequences encoding, e.g., NCSM polypeptides, soluble NSCM-ECD polypeptides, or NCSM-ECD-Ig fusion proteins, or WT hB7-1, or fragments of any of these.

In some embodiments, a recombinant or shuffled promoter having an optimized expression for a particular use with NCSM molecules is utilized. For example, in some therapeutic and/or prophylactic methods or applications, where a lower level expression of a CD28BP or CTLA-4BP is desired (than is typically obtained with a CMV promoter, such as a WT human CMV promoter), at least one recombinant or chimeric CMV promoter nucleotide sequence that is optimized to provide for reduced or suppressed expression levels of the NCSM and/or one or more associated antigens is used. Such

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promoter(s) is operably linked in an expression vector to either or both the NCSM polynucleotide and/or one or more associated antigens (e.g., cancer antigen, such as EpCam/KSA or mutant, variant or derivative of EpCam/KSA). In other embodiments, one or more recombinant, mutant, or chimeric CMV promoters optimized for the particular

5   application can be used, where differential expression between a NCSM polypeptide and at least one associated antigen in one or more vectors is desired (e.g., where it is desirable to express varying amounts of various NCSM polypeptide molecules or co-stimulatory molecules, since their respective concentrations influence or affect one another, and/or where it is desirable to express a comparably higher level of at least one antigen for

10   effective treatment). For example, in some applications, a low expression level of a NCSM polypeptide and a relatively higher expression level of antigen is desired, since it may be particularly useful for successful therapeutic or prophylactic treatment of a particular condition or disease.

Additional Expression Elements

15   Specific initiation signals can aid in efficient translation of a NCSM polynucleotide coding sequence and/or fragments thereof. These signals can include, e.g., the ATG initiation codon and adjacent sequences. In cases where a NCSM coding sequence, its initiation codon and upstream sequences are inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in

20   cases where only coding sequence (e.g., a mature protein coding sequence), or a portion thereof, is inserted, exogenous nucleic acid transcriptional control signals including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The

25   efficiency of expression can enhanced by the inclusion of enhancers appropriate to the cell system in use (see, e.g., Scharf D. et al. (1994) Results Probl Cell Differ 20:125-62; and Bittner et al. (1987) Methods in Enzymol 153:516-544).

Secretion/Localization Sequences

Polynucleotides of the invention encoding NCSM polypeptides and

30   fragments thereof can also be fused, for example, in-frame to nucleic acid encoding a secretion/localization sequence, to target polypeptide expression to a desired cellular compartment, membrane, or organelle, or to direct polypeptide secretion to the periplasmic space or into the cell culture media. Such sequences are known to those of skill, and

include secretion leader or signal peptides, organelle targeting sequences (e.g., nuclear localization sequences, ER retention signals, mitochondrial transit sequences, chloroplast transit sequences), membrane localization/anchor sequences (e.g., stop transfer sequences, GPI anchor sequences), and the like.

5   Expression Hosts
        In a further embodiment, the present invention relates to host cells containing any of the above-described nucleic acids, vectors, or other constructs of the invention. The host cell can be a eukaryotic cell, such as a mammalian cell, a yeast cell, or a plant cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct
10   into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, electroporation, gene or vaccine gun, injection, or other common techniques (see, e.g., Davis, L., Dibner, M., and Battey, I. (1986) Basic Methods in Molecular Biology) for in vivo, ex vivo or in vitro methods.

        A host cell strain is optionally chosen for its ability to modulate the
15   expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the protein include, but are not limited to, acetylation, carboxylation, pegylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing which cleaves a "pre" or a "prepro" form of the protein may also be important for correct insertion, folding and/or function. Different host cells such as *E. coli*,
20   *Bacillus* sp., yeast or mammalian cells such as CHO, HeLa, BHK, MDCK, HEK 293, WI38, etc. have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced foreign protein.

        For long-term, high-yield production of recombinant proteins, stable
25   expression can be used. For example, cell lines which stably express a polypeptide of the invention are transduced using expression vectors which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to
30   confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. For example, resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type.

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Host cells transformed with a nucleotide sequence encoding a NCSM polypeptide or fragments thereof of the invention are optionally cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein or fragment thereof produced by a recombinant cell may be secreted, membrane-bound, or contained intracellularly, depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides encoding mature NCSM polypeptides of the invention can be designed with signal sequences which direct secretion of the mature polypeptides through a prokaryotic or eukaryotic cell membrane.

The present invention also includes at least one NCSM polynucleotide consensus sequence derived from a comparison of two or more NCSM polynucleotide sequences described herein (including, e.g., a polynucleotide encoding a CD28BP or CTLA-4BP of the invention or fragment (e.g., ECD or trunECD) thereof). The present invention also includes at least one NCSM polynucleotide consensus sequence derived from a comparison of two or more NCSM polynucleotide sequences described herein. A NCSM polynucleotide consensus sequence as used herein means a nonnaturally-occurring or recombinant NCSM polynucleotide sequence that predominantly includes those nucleic acid residues that are common to all NCSM polynucleotides of the present invention described herein and that includes, at one or more of those positions wherein there is no nucleic acid residue common to all subtypes, a nucleic acid residue that predominantly occurs at that position and in no event includes any nucleic acid residue which is not extant in that position in at least one NCSM polynucleotide of the invention.

Additional Sequences

The NCSM polypeptide-encoding polynucleotides of the present invention optionally comprise a coding sequence or fragment thereof fused in-frame to a marker sequence which, e.g., facilitates purification of the encoded polypeptide. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, a sequence which binds glutathione (e.g., GST), a hemagglutinin (HA) tag (corresponding to an epitope derived from the influenza hemagglutinin protein; Wilson, I. et al. (1984) Cell 37:767), maltose binding protein sequences, the FLAG epitope utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, WA), and the like. The

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inclusion of a protease-cleavable polypeptide linker sequence between the purification domain and the NCSM sequence is useful to facilitate purification.

For example, one expression vector possible to use in the compositions and methods described herein provides for expression of a fusion protein comprising a polypeptide of the invention fused to a polyhistidine region separated by an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography, as described in Porath et al. (1992) Protein Expression and Purification 3:263-281) while the enterokinase cleavage site provides a method for separating the NCSM polypeptide from the fusion protein. pGEX vectors (Promega; Madison, WI) are optionally used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to ligand-agarose beads (e.g., glutathione-agarose in the case of GST-fusions) followed by elution in the presence of free ligand.

An additional construction in the compositions and methods described herein provides for soluble proteins, and their encoding nucleic acids, comprising NCSM polypeptides (or one or more fragments thereof), e.g., as described herein fused to an Ig molecule, e.g., human IgG Fc ("fragment crystallizable," or fragment complement binding) hinge, CH2 domain and CH3 domain (and nucleotide sequences encoding them). Fc is the portion of the antibody responsible for binding to antibody receptors on cells and the C1q component of complement. Also included are soluble forms of the NCSM polypeptides that comprise secreted forms of the NSCM polypeptides, as produced by chemical synthesis or, e.g., by introducing a plasmid encoding a secreted form of the NCSM polypeptide into a eukaryotic cell. These expressed or secreted soluble NCSM polypeptides or fragments thereof, as well as the soluble NCSM fusion proteins (e.g., NCSM-ECD-Ig fusion proteins or NCSM-truncated-ECD-Ig fusion proteins) or fragments thereof and their encoding nucleic acids are optionally useful as prophylactic and/or therapeutic drugs or as diagnostic tools (see also, e.g., Challita-Eid, P. et al. (1998) J Immunol 160:3419-3426; Sturmhoefel, K. et al. (1999) Cancer Res 59:4964-4972).

Polypeptide Production and Recovery

Following transduction of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means,

and the resulting crude extract retained for further purification. Eukaryotic or microbial cells employed in expression of the NCSM proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, or other methods, which are well know to those skilled in the art.

5   As noted, many references are available for the culture and production of many cells, including cells of bacterial, plant, animal (especially mammalian) and archebacterial origin. See, e.g., Sambrook, Ausubel, and Berger (all *supra*), as well as Freshney (1994) Culture of Animal Cells, a Manual of Basic Technique, third edition, Wiley-Liss, New York and the references cited therein; Doyle and Griffiths (1997)

10   Mammalian Cell Culture: Essential Techniques John Wiley and Sons, NY; Humason (1979) Animal Tissue Techniques, fourth edition W.H. Freeman and Company; and Ricciardelli et al. (1989) In vitro Cell Dev Biol 25:1016-1024. For plant cell culture and regeneration see, e.g., Payne et al. (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc. New York, NY; Gamborg and Phillips (eds.) (1995) Plant Cell, Tissue and Organ

15   Culture; Fundamental Methods Springer Lab Manual, Springer-Verlag (Berlin Heidelberg New York) and Plant Molecular Biology (1993) R.R.D. Croy (ed.) Bios Scientific Publishers, Oxford, U.K. ISBN 0 12 198370 6. Cell culture media in general are set forth in Atlas and Parks (eds.) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, FL. Additional information for cell culture is found in available commercial

20   literature such as the Life Science Research Cell Culture Catalogue (1998) from Sigma-Aldrich, Inc (St Louis, MO) ("Sigma-LSRCCC") and, e.g., the Plant Culture Catalogue and supplement (1997) also from Sigma-Aldrich, Inc (St Louis, MO) ("Sigma-PCCS").

Polypeptides of the invention can be recovered and purified from recombinant cell cultures by any of a number of methods well known in the art, including

25   ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography (e.g., using any of the tagging systems noted herein), hydroxylapatite chromatography, and lectin chromatography. Protein refolding steps can be used, as desired, in completing configuration of the mature NCSM protein or

30   fragments thereof. Finally, high performance liquid chromatography (HPLC) can be employed in the final purification steps. In addition to the references noted, *supra*, a variety of purification methods are well known in the art, including, e.g., those set forth in Sandana (1997) Bioseparation of Proteins, Academic Press, Inc.; Bollag et al. (1996) Protein

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Methods, 2nd Edition Wiley-Liss, NY; Walker (1996) The Protein Protocols Handbook Humana Press, NJ; Harris and Angal (1990) Protein Purification Applications: A Practical Approach IRL Press at Oxford, Oxford, England; Harris and Angal Protein Purification Methods: A Practical Approach IRL Press at Oxford, Oxford, England; Scopes (1993)

5   Protein Purification: Principles and Practice 3rd Edition Springer Verlag, NY; Janson and Ryden (1998) Protein Purification: Principles, High Resolution Methods and Applications, Second Edition Wiley-VCH, NY; and Walker (1998) Protein Protocols on CD-ROM Humana Press, NJ.

In Vitro Expression Systems

10   Cell-free transcription/translation systems can also be employed to produce NCSM polypeptides or fragments thereof using DNAs or RNAs of the present invention or fragments thereof. Several such systems are commercially available. A general guide to in vitro transcription and translation protocols is found in Tymms (1995) In vitro Transcription and Translation Protocols: Methods in Molecular Biology Volume 37, Garland Publishing,

15   NY.

Modified Amino Acids

Polypeptides of the invention may contain one or more modified amino acids. The presence of modified amino acids may be advantageous in, for example, (a) increasing polypeptide serum half-life, (b) reducing polypeptide antigenicity, or (c)

20   increasing polypeptide storage stability. Amino acid(s) are modified, for example, co-translationally or post-translationally during recombinant production (e.g., N-linked glycosylation at N-X-S/T motifs during expression in mammalian cells) or modified by synthetic means.

Non-limiting examples of a modified amino acid include a glycosylated

25   amino acid, a sulfated amino acid, a prenlyated (e.g., farnesylated, geranylgeranylated) amino acid, an acetylated amino acid, an acylated amino acid, a PEG-ylated amino acid, a biotinylated amino acid, a carboxylated amino acid, a phosphorylated amino acid, and the like. References adequate to guide one of skill in the modification of amino acids are replete throughout the literature. Example protocols are found in Walker (1998) Protein

30   Protocols on CD-ROM Humana Press, Towata, NJ.

<u>In Vivo Uses and Applications</u>

Polynucleotides or fragments thereof that encode a NCSM polypeptide of the invention, or complements of the polynucleotides (e.g., antisense or ribozyme molecules), are optionally administered to a cell to accomplish a therapeutically useful process or to

5   express a therapeutically useful product. These in vivo applications, including gene therapy, include a multitude of techniques by which gene expression may be altered in cells. Such methods include, for instance, the introduction of genes for expression of, e.g., therapeutically and/or prophylactically useful polypeptides, such as the NCSM polypeptides of the present invention or fragments thereof.

10   <u>In Vivo Polypeptide Expression</u>
Polynucleotides encoding NCSM polypeptides of the invention and fragments thereof are particularly useful for in vivo therapeutic applications, using techniques well known to those skilled in the art. For example, cultured cells are engineered ex vivo with at least one NCSM polynucleotide (DNA or RNA) and/or other

15   polynucleotide sequences encoding, e.g., at least one of an antigen, cytokine, other co-stimulatory molecule, adjuvant, etc., and the like, with the engineered cells then being returned to the patient. Cells may also be engineered in vivo for expression of one or more polypeptides in vivo. including NCSM polypeptides and/or antigenic peptides.

A number of viral vectors suitable for organismal in vivo transduction and

20   expression are known. Such vectors include retroviral vectors (see, e.g., Miller, <u>Curr Top Microbiol Immunol</u> (1992) 158:1-24; Salmons and Gunzburg (1993) <u>Human Gene Therapy</u> 4:129-141; Miller et al. (1994) <u>Methods in Enzymology</u> 217:581-599) and adeno-associated vectors (reviewed in Carter (1992) <u>Curr Opinion Biotech</u> 3:533-539; Muzcyzka (1992) <u>Curr Top Microbiol Immunol</u>. 158:97-129). Other viral vectors that are used include adenoviral

25   vectors, herpes viral vectors and Sindbis viral vectors, as generally described in, e.g., Jolly (1994) <u>Cancer Gene Therapy</u> 1:51-64; Latchman (1994) <u>Molec Biotechnol</u> 2:179-195; and Johanning et al. (1995) <u>Nucl Acids Res</u> 23:1495-1501.

In one aspect, a pox virus vector can be used. The pox viral vector is transfected with a polynucleotide sequence encoding of the NCSM polypeptides (or

30   fragments thereof) of the invention, such as a CD28BP polypeptide, and is useful in prophylactic, therapeutic and diagnostic applications where enhancement of an immune response, such as increased or improved T cell proliferation or activation (or inhibition of an immune response, such as inhibition of T cell proliferation, if, e.g., a polynucleotide

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encoding a CTAL4-BP polypeptide is used) is desired. See viral vectors discussed in, e.g., Berencsi et al., J Infect Dis (2001)183(8):1171-9; Rosenwirth et al., Vaccine 2001 Feb 8;19(13-14):1661-70; Kittlesen et al., J Immunol (2000) 164(8):4204-11; Brown et al. Gene Ther 2000 7(19):1680-9; Kanesa-thasan et al., Vaccine (2000) 19(4-5):483-91; Sten (2000)

5   Drug 60(2):249-71. Compositions comprising such vectors and an acceptable excipient are also a feature of the invention.

     Gene therapy and genetic vaccines provide methods for combating chronic infectious diseases (e.g., HIV infection, viral hepatitis), as well as non-infectious diseases including cancer and some forms of congenital defects such as enzyme deficiencies, and

10   such methods can be employed with NCSM polynucleotides of the invention, including, e.g., vectors and cells comprising such polynucleotides. Several approaches for introducing nucleic acids and vectors into cells in vivo, ex vivo and in vitro have been used and can be employed with NCSM polynucleotides encoding NCSM polypeptides and fragments thereof (including, e.g., ECD domains and fusion proteins), and vectors comprising NCSM

15   sequences. These approaches include liposome based gene delivery (Debs and Zhu (1993) WO 93/24640 and U.S. Pat. No. 5,641,662; Mannino and Gould-Fogerite (1988) BioTechniques 6(7):682-691; Rose, U.S. Pat. No. 5,279,833; Brigham (1991) WO 91/06309; and Felgner et al. (1987) Proc Natl Acad Sci USA 84:7413-7414; Brigham et al. (1989) Am J Med Sci 298:278-281; Nabel et al. (1990) Science 249:1285-1288; Hazinski et

20   al. (1991) Am J Resp Cell Molec Biol 4:206-209; and Wang and Huang (1987) Proc Natl Acad Sci USA 84:7851-7855); adenoviral vector mediated gene delivery, e.g., to treat cancer (see, e.g., Chen et al. (1994) Proc Natl Acad Sci USA 91:3054-3057; Tong et al. (1996) Gynecol Oncol 61:175-179; Clayman et al. (1995) Cancer Res. 5:1-6; O'Malley et al. (1995) Cancer Res 55:1080-1085; Hwang et al. (1995) Am J Respir Cell Mol Biol 13:7-

25   16; Haddada et al. (1995) Curr Top Microbiol Immunol. 1995 (Pt. 3):297-306; Addison et al. (1995) Proc Natl Acad Sci USA 92:8522-8526; Colak et al. (1995) Brain Res 691:76-82; Crystal (1995) Science 270:404-410; Elshami et al. (1996) Human Gene Ther 7:141-148; Vincent et al. (1996) J Neurosurg 85:648-654), and many others. Replication-defective retroviral vectors harboring therapeutic polynucleotide sequence as part of the retroviral

30   genome have also been used, particularly with regard to simple MuLV vectors. See, e.g., Miller et al. (1990) Mol Cell Biol 10:4239 (1990); Kolberg (1992) J NIH Res 4:43, and Cornetta et al. (1991) Hum Gene Ther 2:215). Nucleic acid transport coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) J Biol Chem, 263:14621-

14624) has also been used. Naked DNA expression vectors have also been described (Nabel et al. (1990), *supra*); Wolff et al. (1990) <u>Science</u>, 247:1465-1468). In general, these approaches can be adapted to the invention by incorporating nucleic acids encoding the NCSM polypeptides or fragments thereof herein into the appropriate vectors.

5   General texts which describe gene therapy protocols, which can be adapted to the present invention by introducing the nucleic acids of the invention into patients, include, e.g., Robbins (1996) <u>Gene Therapy Protocols</u>, Humana Press, NJ, and Joyner (1993) <u>Gene Targeting: A Practical Approach</u>, IRL Press, Oxford, England.

Antisense Technology

10   In addition to expression of the NCSM nucleic acids of the invention as gene replacement nucleic acids, the nucleic acids are also useful for sense and anti-sense suppression of expression, e.g., to down-regulate expression of a nucleic acid of the invention, once, or when, expression of the nucleic acid is no-longer desired in the cell. Similarly, the nucleic acids of the invention, or subsequences or anti-sense sequences

15   thereof, can also be used to block expression of naturally occurring homologous nucleic acids. A variety of sense and anti-sense technologies are known in the art, e.g., as set forth in Lichtenstein and Nellen (1997) <u>Antisense Technology: A Practical Approach</u> IRL Press at Oxford University, Oxford, England, and in Agrawal (1996) <u>Antisense Therapeutics</u> Humana Press, NJ, and the references cited therein.

20   ### Use as Probes
Also contemplated are uses of polynucleotides, also referred to herein as oligonucleotides, typically having at least 12 bases, preferably at least 15, more preferably at least 20, at least 30, or at least 50 or more bases, which hybridize under highly stringent conditions to a NCSM polynucleotide, variant or homologue sequence described herein or

25   fragments thereof. The polynucleotides may be used as probes, primers, sense and antisense agents, and the like, according to methods as noted *supra*.

SEQUENCE VARIATIONS

Silent Variations
Because of the degeneracy of the genetic code, a large number of

30   functionally identical nucleic acids encode any given polypeptide. For instance, inspection of the codon table (Table 1) shows that codons AGA, AGG, CGA, CGC, CGG, and CGU all encode the amino acid arginine. Thus, at every position in a nucleic acid sequence

where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described above without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" are one species of "conservatively modified variations." It is understood that U in an RNA sequence corresponds to T in a DNA

5 sequence.

Table 1
Codon Table

Amino acids			Codon					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

10 It will thus be appreciated by those skilled in the art that due to the degeneracy of the genetic code, a multitude of nucleic acids sequences encoding NCSM polypeptides of the invention may be produced, some of which may bear minimal sequence homology to the nucleic acid sequences explicitly disclosed herein. Using, as an example, the nucleic acid sequence corresponding to nucleotides 1-15 of SEQ ID NO:1, ATG GGT

15 CAC ACA ATG, a silent variation of this sequence includes ATG GGA CAT ACG ATG, both of which sequences encode the amino acid sequence MGHTM, which corresponds to amino acids 1-5 of SEQ ID NO:48.

One of ordinary skill in the art will recognize that each codon in a nucleic acid (except AUG and UGC, which are ordinarily the only codon for methionine and

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tryptophan, respectively) can be modified by standard techniques to encode a functionally identical polypeptide. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in any described sequence. The invention also provides each and every possible variation of a nucleic acid sequence encoding a NCSM polypeptide of the invention that can be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code (codon) (e.g., as set forth in Table 1), as applied to the nucleic acid sequence encoding a polypeptide of the invention or fragment thereof. All such variations of every nucleic acid herein are specifically provided and described by consideration of the sequence in combination with the genetic code. One of skill is fully able to generate any silent substitution of the sequences listed herein. For example, the invention includes polynucleotides comprising one or more silent variations of any polynucleotide sequence selected from SEQ ID NOS:1-21 and 95-142, or complementary polynucleotides thereof. Also included are polynucleotides comprising one or more silent variations of a nucleotide segment or fragment of any polynucleotide sequence selected from SEQ ID NOS:1-21 and 95-142, or complementary polynucleotides thereof, wherein such nucleotide segment or fragment comprises a nucleotide sequence that encodes a signal peptide and/or extracellular domain of an NCSM polypeptide or a nucleotide sequence that encodes an extracellular domain of an NCSM polypeptide. In one such aspect, for example, polynucleotides comprising one or more silent variations of a nucleotide segment or fragment comprising nucleic acid residues 1-102 (encoding a signal peptide) and/or 103-729 (encoding extracellular domain) of any of SEQ ID NOS:1-21 and 95-142 are provided. Also provided are polynucleotides comprising one or more silent variations of any polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:48-68, 174,221, 283-295, 290-293, or complementary polynucleotide thereof, and nucleotide segments fragments thereof, including those encoding an NCSM signal peptide and/or extracellular domain. Also provided are polypeptides encoded by all such polynucleotides of the invention comprising one or more silent variations.

Conservative Variations

"Conservatively modified variations," or simply "conservative variations," of a particular nucleic acid sequence refer to those nucleic acid sequences that encode identical or essentially identical amino acid sequences, or, where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. One of skill will recognize that

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individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 4%, 2% or 1%) in an encoded sequence of the invention are "conservatively modified variations" where the alterations result in the deletion, addition, and/or substitution of an

5   amino acid with a chemically similar amino acid.

Conservative substitution tables providing functionally similar amino acids are well known in the art. Table 2 sets forth six exemplary groups that contain amino acids that are "conservative substitutions" for one another.

Table 2

10   Conservative Substitution Groups

1	Alanine (A)	Serine (S)	Threonine (T)	
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Lysine (K)		
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

Additional groups of amino acids can also be formulated. For example, amino acids can be grouped by similar function or chemical structure or composition (e.g.,

15   acidic, basic, aliphatic, aromatic, sulfur-containing). For example, an aliphatic grouping may comprise: Glycine (G), Alanine, Valine, Leucine, Isoleucine. Other groups containing amino acids that are conservative substitutions for one another include: Aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); Sulfur-containing: Methionine (M), Cysteine (C); Basic: Arginine (R), Lysine (K), Histidine (H); Acidic: Aspartic acid (D),

20   Glutamic acid (E), Asparagine (N), Glutamine (Q). See also Creighton (1984) Proteins, W.H. Freeman and Company, for additional groupings of amino acids.

Thus, "conservatively substituted variations" of a polypeptide sequence of the present invention include substitutions of a small percentage, typically less than 5%, more typically less than 4%, 3%, 2%, or 1%, of the amino acids of the sequence, with a

25   conservatively selected amino acid of the same conservative substitution group.

For example, a conservatively substituted variation of the polypeptide identified herein as SEQ ID NO:48 may contain "conservative substitutions," according to the six groups defined above, in up to 15 residues (i.e., 5% of the amino acids) in the 296

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amino acid polypeptide. Listing of a polypeptide or protein sequence herein, in conjunction with the above substitution table, provides an express listing of all conservatively substituted polypeptide or protein sequences.

In a further example, if four conservative substitutions were localized in the region corresponding to amino acids 69-94 of SEQ ID NO:48, examples of conservatively substituted variations of this region, QKDSK MVLAI LPGKV QVWPE YKNRTI, would include:

NKDSK MVVAI LPGKV QVFPE YKNKTI and

QKDAK MVLAI LPGRV QMWPE YKQRTI and the like, where conservative substitutions listed in Table 2 (in the above example, conservative substitutions are underlined).

The addition of one or more nucleic acids or sequences that do not alter the encoded activity of a nucleic acid molecule of the invention, such as the addition of a non-functional sequence, is a conservative variation of the basic nucleic acid molecule, and the addition of one or more amino acid residues that do not alter the activity of a polypeptide of the invention is a conservative variation of the basic polypeptide. Both such types of additions are features of the invention.

One of skill will appreciate that many conservative variations of the nucleic acid sequence constructs that are disclosed yield a functionally identical construct. For example, as discussed above, owing to the degeneracy of the genetic code, "silent substitutions" (i.e., substitutions in a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of *every* nucleic acid sequence that encodes an amino acid. Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties, are also readily identified as being highly similar to a disclosed construct. Such conservative variations of each disclosed sequence are a feature of the present invention.

In one aspect, the invention includes polynucleotides comprising one or more conservative variations of any polypeptide selected from SEQ ID NOS:48-68, 174-221, 283-285, and 290-293. Also included are polypeptides comprising one or more conservative variations of a polypeptide segment or fragment of any polypeptide sequence selected from SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, wherein such polypeptide segment or fragment comprises an amino acid sequence comprising a signal

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peptide and/or ECD of an NCSM polypeptide or an amino acid sequence comprising an ECD of an NCSM polypeptide. In one such aspect, polypeptides comprising at least one conservative variation of a polypeptide segment or fragment comprising amino acid residues 1-34 (encoding a signal peptide) and/or 35-243 (encoding an ECD) of any of SEQ

5   ID NOS: 48-68, 174-221, 283-285, and 290-293 are provided. Also provided are polypeptides comprising at least one conservative variation of any polypeptide sequence encoded by a polynucleotide sequence selected from SEQ ID NOS:1-21 and 95-142, or complementary polynucleotide thereof, and nucleotide segments fragments thereof, including those encoding an NCSM signal peptide and/or ECD. Also provided are

10   polynucleotides encoded by all such polynucleotides of the invention comprising one or more conservative variations.

Nucleic Acid Hybridization

Nucleic acids "hybridize" when they associate, typically in solution. Nucleic acids hybridize due to a variety of well characterized physico-chemical forces, such as

15   hydrogen bonding, solvent exclusion, base stacking and the like. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, part I, chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," (Elsevier, New York) (hereinafter "Tjissen"), as well as in Ausubel, *supra*, Hames

20   and Higgins (1995) Gene Probes 1, IRL Press at Oxford University Press, Oxford, England (Hames and Higgins 1) and Hames and Higgins (1995) Gene Probes 2, IRL Press at Oxford University Press, Oxford, England (Hames and Higgins 2) provide details on the synthesis, labeling, detection and quantification of DNA and RNA, including oligonucleotides.

An indication that two nucleic acid sequences are substantially identical is

25   that the two molecules hybridize to each other under at least stringent conditions. The phrase "hybridizing specifically to," refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a

30   target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence.

"Stringent hybridization wash conditions" and "stringent hybridization conditions" in the context of nucleic acid hybridization experiments, such as Southern and northern hybridizations, are sequence dependent, and are different under different environmental parameters. An extensive guide to hybridization of nucleic acids is found in

5   Tijssen (1993), *supra*, and in Hames and Higgins 1 and Hames and Higgins 2, *supra*.

For purposes of the present invention, generally, "highly stringent" hybridization and wash conditions are selected to be about 5° C (or less) lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH (as noted below, highly stringent conditions can also be referred to in comparative terms). The

10   T_m is the temperature (under defined ionic strength and pH) at which 50% of the test sequence hybridizes to a perfectly matched probe. In other words, the T_m indicates the temperature at which the nucleic acid duplex is 50% denatured under the given conditions and its represents a direct measure of the stability of the nucleic acid hybrid. Thus, the T_m corresponds to the temperature corresponding to the midpoint in transition from helix to

15   random coil; it depends on length, nucleotide composition, and ionic strength for long stretches of nucleotides. Typically, under "stringent conditions," a probe will hybridize to its target subsequence, but to no other sequences. "Very stringent conditions" are selected to be equal to the T_m for a particular probe.

After hybridization, unhybridized nucleic acid material can be removed by a

20   series of washes, the stringency of which can be adjusted depending upon the desired results. Low stringency washing conditions (e.g., using higher salt and lower temperature) increase sensitivity, but can product nonspecific hybridization signals and high background signals. Higher stringency conditions (e.g., using lower salt and higher temperature that is closer to the hybridization temperature) lowers the background signal, typically with only

25   the specific signal remaining. See, Rapley, R. and Walker, J.M. eds., Molecular Biomethods Handbook (Humana Press, Inc. 1998) (hereinafter "Rapley and Walker"), which is incorporated herein by reference in its entirety for all purposes.

The T_m of a DNA-DNA duplex can be estimated using equation (1):

$$T_m (°C) = 81.5°C + 16.6 (\log_{10}M) + 0.41 (\%G + C) - 0.72 (\%f) - 500/n,$$

30   where M is the molarity of the monovalent cations (usually Na+), (%G + C) is the percentage of guanosine (G) and cystosine (C) nucleotides, (%f) is the percentage of formalize and n is the number of nucleotide bases (i.e., length) of the hybrid. See, Rapley and Walker, *supra*.

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The T_m of an RNA-DNA duplex can be estimated using equation (2):

$$T_m \ (^\circ C) = 79.8^\circ C + 18.5 \ (\log_{10}M) + 0.58 \ (\%G + C) - 11.8(\%G + C)^2 - 0.56$$

$$(\%f) - 820/n,$$

where M is the molarity of the monovalent cations (usually Na+), (%G +

5   C)is the percentage of guanosine (G) and cystosine (C) nucleotides, (%f) is the percentage

of formamide and n is the number of nucleotide bases (i.e., length) of the hybrid. Id.

Equations 1 and 2 above are typically accurate only for hybrid duplexes longer than about

100-200 nucleotides. Id.

The Tm of nucleic acid sequences shorter than 50 nucleotides can be

10   calculated as follows:

$$T_m \ (^\circ C) = 4(G + C) + 2(A + T),$$ where A (adenine), C, T (thymine), and G

are the numbers of the corresponding nucleotides.

An example of stringent hybridization conditions for hybridization of

complementary nucleic acids which have more than 100 complementary residues on a filter

15   in a Southern or northern blot is 50% formalin (or formamide) with 1 mg of heparin at

42°C, with the hybridization being carried out overnight. An example of stringent wash

conditions is a 0.2x SSC wash at 65°C for 15 minutes (see Sambrook, *supra*, for a

description of SSC buffer). Often, the high stringency wash is preceded by a low stringency

wash to remove background probe signal. An example low stringency wash is 2x SSC at

20   40°C for 15 minutes. An example of highly stringent wash conditions is 0.15M NaCl at

72°C for about 15 minutes. An example medium stringency wash for a duplex of, e.g.,

more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency

wash for a duplex of, e.g., more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes.

For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve

25   salt concentrations of less than about 1.0 M Na$^+$ ion, typically about 0.01 to 1.0 M Na$^+$ ion

concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about

30°C. Stringent conditions can also be achieved with the addition of destabilizing agents

such as formamide.

In general, a signal to noise ratio of 2x or 2.5x-5x (or higher) than that

30   observed for an unrelated probe in the particular hybridization assay indicates detection of a

specific hybridization. Detection of at least stringent hybridization between two sequences

in the context of the present invention indicates relatively strong structural similarity or

homology to, e.g., the nucleic acids of the present invention provided in the sequence listings herein.

As noted, "highly stringent" conditions are selected to be about 5° C or less lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Target sequences that are closely related or identical to the nucleotide sequence of interest (e.g., "probe") can be identified under highly stringency conditions. Lower stringency conditions are appropriate for sequences that are less complementary. See, e.g., Rapley and Walker; Sambrook, all *supra*.

Comparative hybridization can be used to identify nucleic acids of the invention, and this comparative hybridization method is a preferred method of distinguishing nucleic acids of the invention. Detection of highly stringent hybridization between two nucleotide sequences in the context of the present invention indicates relatively strong structural similarity/homology to, e.g., the nucleic acids provided in the sequence listing herein. Highly stringent hybridization between two nucleotide sequences demonstrates a degree of similarity or homology of structure, nucleotide base composition, arrangement or order that is greater than that detected by stringent hybridization conditions. In particular, detection of highly stringent hybridization in the context of the present invention indicates strong structural similarity or structural homology (e.g., nucleotide structure, base composition, arrangement or order) to, e.g., the nucleic acids provided in the sequence listings herein. For example, it is desirable to identify test nucleic acids which hybridize to the exemplar nucleic acids herein under stringent conditions.

Thus, one measure of stringent hybridization is the ability to hybridize to one of the listed nucleic acids of the invention (e.g., nucleic acid sequences SEQ ID NOS:1-47, 95-173, and 253-262, and complementary polynucleotide sequences thereof) under highly stringent conditions (or very stringent conditions, or ultra-high stringency hybridization conditions, or ultra-ultra high stringency hybridization conditions). Stringent hybridization (including, e.g., highly stringent, ultra-high stringency, or ultra-ultra high stringency hybridization conditions) and wash conditions can easily be determined empirically for any test nucleic acid.

For example, in determining highly stringent hybridization and wash conditions, the hybridization and wash conditions are gradually increased (e.g., by increasing temperature, decreasing salt concentration, increasing detergent concentration and/or increasing the concentration of organic solvents, such as formalin, in the

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hybridization or wash), until a selected set of criteria are met. For example, the hybridization and wash conditions are gradually increased until a probe comprising one or more nucleic acid sequences selected from SEQ ID NOS:1-47, 95-173, and 253-262, and complementary polynucleotide sequences thereof, binds to a perfectly matched

5   complementary target (again, a nucleic acid comprising one or more nucleic acid sequences selected from SEQ ID NOS:1-47, 95-173, and 253-262, and complementary polynucleotide sequences thereof), with a signal to noise ratio that is at least 2.5x, and optionally 5x or more as high as that observed for hybridization of the probe to an unmatched target. In this case, the unmatched target is a nucleic acid corresponding to, e.g., a known B7-1 or related

10   known co-stimulatory homologue or the like, e.g., a B7-1 nucleic acid (other than those in the accompanying sequence listing) present in a public database such as GenBank™ at the time of filing of the subject application. Examples of such unmatched target nucleic acids include, e.g., the following: A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896,

15   AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958,

20   Y08823, and Y09950, where the numbers correspond to GenBank accession numbers. Additional such sequences can be identified in GenBank by one of ordinary skill in the art.

A test nucleic acid is said to specifically hybridize to a probe nucleic acid when it hybridizes at least ½ as well to the probe as to the perfectly matched complementary target, i.e., with a signal to noise ratio at least ½ as high as hybridization of the probe to the

25   target under conditions in which the perfectly matched probe binds to the perfectly matched complementary target with a signal to noise ratio that is at least about 2.5x-10x, typically 5x-10x as high as that observed for hybridization to any of the unmatched target nucleic acids such as, A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519,

30   AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593,

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U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950 (where the numbers correspond to GenBank accession numbers), or, e.g., other similar known B7-1 or related co-stimulatory sequences or the like presented in GenBank.

In one aspect, the invention provides a target nucleic acid that, but for the degeneracy of the genetic code, hybridizes under stringent conditions to a unique coding oligonucleotide that encodes a unique subsequence in a polypeptide selected from SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, where the unique subsequence is unique compared to a polypeptide encoded by any of above GenBank Nucleotide Access Nos. For some such nucleic acids, the stringent conditions are selected such that a perfectly complementary oligonucleotide to the coding oligonucleotide hybridizes to the coding oligonucleotide with at least about a 5x higher signal to noise ratio than for hybridization of the perfectly complementary oligonucleotide to a control nucleic acid corresponding to any of GenBank Nucleotide Accession Nos. set forth above.

Ultra high-stringency hybridization and wash conditions are those in which the stringency of hybridization and wash conditions are increased until the signal to noise ratio for binding of the probe to the perfectly matched complementary target nucleic acid is at least 10x as high as that observed for hybridization to any of the unmatched target nucleic acids, such as, A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950 (where the numbers correspond to GenBank accession numbers), or, e.g., to other similar known B7-1 or co-stimulatory molecule sequences or the like presented in GenBank. A target nucleic acid which hybridizes to a probe under such conditions, with a signal to noise ratio of at least ½ that of the perfectly matched complementary target nucleic acid is said to bind to the probe under ultra-high stringency conditions.

Similarly, even higher levels of stringency can be determined by gradually increasing the hybridization and/or wash conditions of the relevant hybridization assay. For example, those in which the stringency of hybridization and wash conditions are increased until the signal to noise ratio for binding of the probe to the perfectly matched

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complementary target nucleic acid is at least 10x, 20X, 50X, 100X, or 500X or more as high as that observed for hybridization to any of the unmatched target nucleic acids, such as those represented by: A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519,

5   AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823,

10   and Y09950 (where the numbers correspond to GenBank accession numbers), or, e.g., other similar B7-1 or co-stimulatory sequences or the like presented in GenBank can be identified. A target nucleic acid which hybridizes to a probe under such conditions, with a signal to noise ratio of at least ½ that of the perfectly matched complementary target nucleic acid is said to bind to the probe under ultra-ultra-high stringency conditions.

15   Target nucleic acids which hybridize to the nucleic acids represented by SEQ ID NOS:1-47, 95-173, and 253-262 under high, ultra-high and ultra-ultra high stringency conditions are a feature of the invention. Examples of such nucleic acids include those with one or a few silent or conservative nucleic acid substitutions as compared to a given nucleic acid sequence.

20   Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code, or when antisera generated against one or more of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, which has been subtracted

25   using the polypeptides encoded by known or existing B7-1 or similar or related co-stimulatory sequences or the like, including, e.g., those encoded by the following: A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834,

30   AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950 (where the

numbers correspond to GenBank accession numbers), or, e.g., other similar B7-1, co-stimulatory sequences, or the like presented in, e.g., GenBank. Further details on immunological identification of polypeptides of the invention are found below. Additionally, for distinguishing between duplexes with sequences of less than about 100

5   nucleotides, a TMAC1 hybridization procedure known to those of skill in the art can be used. See, e.g., Sorg, U. et al. 1 Nucleic Acids Res. (Sept. 11, 1991) 19(17), incorporated herein by reference in its entirety for all purposes.

In one aspect, the invention provides a nucleic acid which comprises a unique subsequence in a nucleic acid selected from any of SEQ ID NOS:1-47, 95-173, and

10   253-262. The unique subsequence is unique as compared to a nucleic acid corresponding to any of, e.g., A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762,

15   AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950 (where the numbers correspond to GenBank accession numbers), or, e.g., other similar B7-1 or co-stimulatory sequences or the like presented in GenBank. Such unique subsequences

20   can be determined by aligning any of SEQ ID NOS:1-47, 95-173, and 253-262 against the complete set of nucleic acids, e.g., those corresponding to, e.g., A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442,

25   AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950, or other sequences available, e.g., in a public database, at the filing date of the subject application. Alignment can be

30   performed using the BLAST algorithm set to default parameters. Any unique subsequence is useful, e.g., as a probe to identify the nucleic acids of the invention.

Similarly, the invention includes a polypeptide which comprises a unique amino acid subsequence in a polypeptide selected from any of SEQ ID NOS:48-94, 174-

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252, 263-272, and 283-293. Here, the unique subsequence is unique as compared to a polypeptide or amino acid sequence corresponding to, e.g., any of A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950 (where the numbers correspond to GenBank accession numbers). Here again, the polypeptide is aligned against the existing polypeptides (the control polypeptides). Note that where the sequence corresponds to a non-translated sequence such as a pseudo-gene, the corresponding polypeptide is generated simply by in silico translation of the nucleic acid sequence into an amino acid sequence, where the reading frame is selected to correspond to the reading frame of homologous NCSM nucleic acids. Such polypeptides are optionally made by synthetic or recombinant approaches, or can even be ordered from companies specializing in polypeptide production.

In addition, the present invention provides a target nucleic acid which, but for codon degeneracy, hybridizes under at least stringent or highly stringent conditions (or conditions of greater stringency) to a unique coding oligonucleotide which encodes a unique subsequence in a polypeptide selected from any of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, wherein the unique subsequence is unique as compared to a an amino acid subsequence of a known B7-1 or related co-stimulatory polypeptide sequence or the like shown in GenBank or to a polypeptide corresponding to any of the control polypeptides. Unique sequences are determined as noted above.

In one example, the stringent conditions are selected such that a perfectly complementary oligonucleotide to the coding oligonucleotide hybridizes to the coding oligonucleotide with at least about a 5-10x higher signal to noise ratio than for hybridization of the perfectly complementary oligonucleotide to a control nucleic acid corresponding to any of the control polypeptides. Conditions can be selected such that higher ratios of signal to noise are observed in the particular assay that is used, e.g., about 15x, 20x, 30x, 50x or more. In this example, the target nucleic acid hybridizes to the unique coding oligonucleotide with at least a 2x higher signal to noise ratio as compared to hybridization of the control nucleic acid to the coding oligonucleotide. Again, higher signal to noise

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ratios can be selected, e.g., about 2.5x, about 5x, about 10x, about 20x, about 30x, about 50x or more. The particular signal depends on the label used in the relevant assay, e.g., a fluorescent label, colorimetric label, radio active label, or the like.

In another aspect, the invention provides a polypeptide comprising a unique subsequence in a polypeptide selected from any of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, wherein the unique subsequence is unique as compared to a polypeptide sequence corresponding to a known B7-1, co-stimulatory polypeptide or the like, such as, e.g., a B7-1 or co-stimulatory polypeptide sequence present in GenBank.

Percent Sequence Identity – Sequence Similarity

The degree to which one nucleic acid is similar to another provides an indication of whether there is an evolutionary relationship between the two or more nucleic acids. In particular, where a high level of sequence identity is observed, it is inferred that the nucleic acids are derived from a common ancestor (i.e., that the nucleic acids are homologous). In addition, sequence similarity implies similar structural and functional properties for the two or more nucleic acids and the sequences they encode. Accordingly, in the context of the present invention, sequences which have a similar sequence to any given exemplar sequence are a feature of the present invention. In particular, sequences that have share percent sequence identities as defined below are a feature of the invention.

A variety of methods of determining sequence relationships can be used, including manual alignment and computer assisted sequence alignment and analysis. This later approach is a preferred approach in the present invention, due to the increased throughput afforded by computer-assisted methods. A variety of computer programs for performing sequence alignment are available, or can be produced by one of skill.

As noted above, the sequences of the nucleic acids and polypeptides (and fragments thereof) employed in the subject invention need not be identical, but can be substantially identical (or substantially similar), to the corresponding sequence of a NCSM polypeptide or nucleic acid molecule (or fragment thereof) or related molecule. For example, the polypeptides can be subject to various changes, such as one or more amino acid or nucleic acid insertions, deletions, and substitutions, either conservative or non-conservative, including where, e.g., such changes might provide for certain advantages in their use, e.g., in their therapeutic or prophylactic use or administration or diagnostic application. The nucleic acids can also be subject to various changes, such as one or more substitutions of one or more nucleic acids in one or more codons such that a particular

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codon encodes the same or a different amino acid, resulting in either a conservative or non-conservative substitution, or one or more deletions of one or more nucleic acids in the sequence. The nucleic acids can also be modified to include one or more codons that provide for optimum expression in an expression system (e.g., mammalian cell or

5   mammalian expression system), while, if desired, said one or more codons still encode the same amino acid(s). Such nucleic acid changes might provide for certain advantages in their therapeutic or prophylactic use or administration, or diagnostic application. The nucleic acids and polypeptides can be modified in a number of ways so long as they comprise a sequence substantially identical (as defined below) to a sequence in a respective

10   NCSM nucleic acid or polypeptide molecule.

Alignment and comparison of relatively short amino acid sequences (less than about 30 residues) is typically straightforward. Comparison of longer sequences can require more sophisticated methods to achieve optimal alignment of two sequences. Optimal alignment of sequences for aligning a comparison window can be conducted by the

15   local homology algorithm of Smith and Waterman (1981) Adv Appl Math 2:482, by the homology alignment algorithm of Needleman and Wunsch (1970) J Mol Biol 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc Natl Acad Sci USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer

20   Group, 575 Science Dr., Madison, WI; and BLAST, see, e.g., Altschul et al. (1977) Nuc Acids Res 25:3389-3402 and Altschul et al. (1990) J Mol Biol 215:403-410), or by inspection, with the best alignment (i.e., resulting in the highest percentage of sequence similarity or sequence identity over the comparison window) generated by the various methods being selected.

25   The term "identical" or percent "identity," in the context of two or more nucleic acid or polypeptide sequences, refers to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

30   The term "sequence identity" or "percent identity" ("% identity") means that two polynucleotide or polypeptide sequences are identical (i.e., on a nucleotide-by-nucleotide basis or amino acid-by-amino acid basis, respectively) over a window of comparison. The term "percentage of sequence identity" (or "percent sequence identity" or

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simply "percent identity" or "% identity") or "percentage of sequence similarity" (or

"percent sequence similarity" or simply "percent similarity") is calculated by comparing

two optimally aligned polynucleotide or polypeptide sequences over the window of

comparison, determining the number of positions at which the identical residues occur in

5   both sequences to yield the number of matched positions, dividing the number of matched

positions by the total number of positions in the window of comparison (i.e., the window

size), and multiplying the result by 100 to yield the percentage of sequence identity (or

percentage of sequence similarity). Thus, for example, with regard to polypeptide

sequences, the term sequence identity means that two polypeptide sequences are identical

10   (on an amino acid-by-amino acid basis) over a window of comparison, and a percentage of

amino acid residue sequence identity (or percentage of amino acid residue sequence

similarity), can be calculated. For sequence comparison, typically one sequence acts as a

reference sequence to which test sequences are compared. When using a sequence

comparison algorithm, test and reference sequences are input into a computer, subsequence

15   coordinates are designated, if necessary, and sequence algorithm program parameters are

designated. The sequence comparison algorithm then calculates the percent sequence

identity for the test sequence(s) relative to the reference sequence, based on the designated

program parameters. Maximum correspondence can be determined by using one of the

sequence algorithms described herein (or other algorithms available to those of ordinary

20   skill in the art) or by visual inspection.

          The phrase "substantially identical" or "substantial identity" in the context of

two nucleic acids or polypeptides, refers to two or more sequences or subsequences that

have at least about 50%, 60%, 70%, 75%, preferably 80% or 85%, more preferably 90%,

91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or more nucleotide or amino

25   acid residue % identity, respectively, when compared and aligned for maximum

correspondence, as measured using one of the following sequence comparison algorithms or

by visual inspection. In certain embodiments, the substantial identity exists over a region of

amino acid sequences that is at least about 50 residues in length, preferably over a region of

at least about 100 residues in length, and more preferably the sequences are substantially

30   identical over at least about 150, 200, or 250 amino acid residues. In certain aspects,

substantial identity exists over a region of nucleic acid sequences of at least about 500

residues, preferably over a region of at least about 600 residues in length, and more

preferably the sequences are substantially identical over at least about 700, 800, or 850

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nucleic acid residues. In some aspects, the amino acid or nucleic acid sequences are substantially identical over the entire length of the corresponding coding region.

As applied to polypeptides and peptides, the term "substantial identity" typically means that two polypeptide or peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights (described in detail below) or by visual inspection, share at least about 60% or 70%, often at least 75%, preferably at least about 80% or 85%, more preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% or more percent amino acid residue sequence identity or sequence similarity. Similarly, as applied in the context of two nucleic acids, the term substantial identity or substantial similarity means that the two nucleic acid sequences, when optimally aligned, such as by the programs BLAST, GAP or BESTFIT using default gap weights (described in detail below) or by visual inspection, share at least about 60 percent, 70 percent, or 80 percent sequence identity or sequence similarity, preferably at least about 90 percent amino acid residue sequence identity or sequence similarity, more preferably at least about 95 percent sequence identity or sequence similarity, or more (including, e.g., about 90, 91, 92, 93, 94, 95, 96, 97, 98, 98.5, 99, 99.5,or more percent nucleotide sequence identity or sequence similarity).

In one aspect, the present invention provides nucleic acids encoding NCSM amino acid molecules (e.g., full-length polypeptide, signal peptide, ECD, cytoplasmic domain, transmembrane domain, mature region, or other fragment) having at least about 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5% or more percent sequence identity or sequence similarity with the nucleic acid of any of SEQ ID NOS:1-47, 95-173, and 253-262 or a fragment thereof, including, e.g., one or more of a signal peptide, ECD, cytoplasmic domain, transmembrane domain, or mature region or any combination thereof. Some such encoded polypeptides have the CD28BP or CTLA-4BP properties described herein.

In another aspect, the present invention provides NCSM polypeptides (e.g., full-length NCSM polypeptide, signal peptide, ECD, cytoplasmic domain, transmembrane domain, mature region, or other fragment), and fusion proteins comprising said polypeptides, having at least about 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more percent sequence identity or sequence similarity with the polypeptide of any of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293 or a fragment thereof , including, e.g., one or more of a signal peptide, ECD,

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cytoplasmic domain, transmembrane domain, or mature region or any combination thereof. Such fragments of SEQ ID NOS:69-92, 222-272, and 286-288 may have at least one CTLA4BP property described herein, such as, e.g., an ability to inhibit T cell proliferation or activation in conjunction with stimulation of T cell receptor (e.g., by antigen or anti-CD3

5   Ab) and/or a CTLA-4/CD28 binding affinity ratio about equal to or greater than that of hB7-1. Such fragments of SEQ ID NOS:48-68, 174-221, 283-285, and 289-293 may have at least one CD28BP property described herein, such as, e.g., an ability to induce T cell proliferation or activation in conjunction with stimulation of T cell receptor (e.g., by antigen or anti-CD3 Ab) and/or a CD28/CTLA-4 binding affinity ratio about equal to or greater

10   than that of hB7-1. Such fragments of SEQ ID NOS:93-94 may have an ability to induce T cell proliferation or activation in conjunction with stimulation of T cell receptor (by, e.g., an antigen) and/or a CD28/CTLA-4 binding affinity ratio approximately equal to that of a primate, such as hB7-1.

          In yet another aspect, the present invention provides NCSM homologue

15   polypeptides that are substantially identical or substantially similar over at least about 150, 180, 170, 190, 200, 210, 225, 230, 240, 250, 275, or 285 or more contiguous amino acids of at least one of SEQ ID NOS:69-92, 222-272, and 286-288; some such polypeptides may have an ability to inhibit T cell proliferation or activation and/or a CTLA-4/CD28 binding affinity ratio about equal to or greater than that of hB7-1 as described herein.

20   In yet another aspect, the present invention provides NCSM homologue polypeptides that are substantially identical or substantially similar over at least about 150, 180, 170, 190, 200, 210, 225, 230, 240, 250, 275, or 285 or more contiguous amino acids of at least one of SEQ ID NOS:48-68, 174-221, 283-285, and 289-293; some such polypeptides may have an ability to induce T cell proliferation or activation in conjunction

25   with stimulation of T cell receptor (e.g., by antigen or anti-CD3 Ab) and/or a CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1.

          NCSM homologue polypeptides that are substantially identical or substantially similar over at least about 150, 180, 170, 190, 200, 210, 225, 230, 240, 250, 275, or 285 or more contiguous amino acids of at least one of SEQ ID NOS:93-94; some

30   such polypeptides may have an ability to induce T cell proliferation or activation in conjunction with stimulation of T cell receptor (by, e.g., anti-CD3 Ab or antigen) and/or a CD28/CTLA-4 binding affinity ratio about equal to that of a primate, such as hB7-1.

A feature of the invention is a NCSM polypeptide comprising at least 175 contiguous amino acids of any one of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293. In other embodiments, the polypeptide comprises about 175, 200, 210, 225, 275, or more contiguous amino acid residues of any one of SEQ ID NOS:48-94, 174-252, 263-272, and

5   283-293. In other embodiments, the polypeptide is at least about 280 amino acids, and still more preferably at least about 285 amino acids in length.

Alternatively, parameters are set such that one or more sequences of the invention are identified by alignment to a query sequence selected from among SEQ ID NOS:49-94, 174-252, 263-272 and 283-293, while sequences corresponding to unrelated

10   polypeptides, e.g., those encoded by known nucleic acid sequences represented by GenBank accession numbers (e.g., known B7-1 sequences) are not identified.

Preferably, residue positions that are not identical differ by conservative amino acid substitutions. Conservative amino acid substitution refers to the interchangeability of residues having similar side chains. For example, a group of amino acids having

15   aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids

20   having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, arginine-lysine-histidine, lysine-arginine, alanine-valine, and asparagine-glutamine.

Alignment and comparison of relatively short amino acid sequences (less than about 30 residues) is typically straightforward. Comparison of longer sequences can

25   require more sophisticated methods to achieve optimal alignment of two sequences. Optimal alignment of sequences for aligning a comparison window can be conducted by the local homology algorithm of Smith and Waterman (1981) Adv Appl Math 2:482, by the homology alignment algorithm of Needleman and Wunsch (1970) J Mol Biol 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc Natl Acad Sci USA

30   85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, with the best alignment (i.e.,

resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods being selected.

A preferred example of an algorithm that is suitable for determining percent sequence identity (percent identity) and sequence similarity is the FASTA algorithm, which is described in Pearson, W.R. & Lipman, D. J. (1988) Proc Natl Acad Sci USA 85:2444. *See* also, W. R. Pearson (1996) Methods Enzymology 266:227-258. Preferred parameters used in a FASTA alignment of DNA sequences to calculate percent identity are optimized, BL50 Matrix 15: -5, k-tuple = 2; joining penalty = 40, optimization = 28; gap penalty -12, gap length penalty =-2; and width = 16.

Other preferred examples of algorithm that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1997) Nuc Acids Res 25:3389-3402 and Altschul et al. (1990) J Mol Biol 215:403-410, respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http: //www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For

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amino acid sequences, the BLASTP program (e.g., BLASTP 2.0.14; Jun-29-2000) uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see*, Henikoff & Henikoff (1989) Proc Natl Acad Sci USA 89:10915) uses alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. Again, as with

5   other suitable algorithms, the stringency of comparison can be increased until the program identifies only sequences that are more closely related to those in the sequence listings herein (i.e., SEQ ID NOS:1-47, 95-173, and 253-262 or, alternatively, SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, rather than sequences that are more closely related to other similar sequences such as, e.g., those nucleic acid sequences represented by GenBank

10   accession numbers set forth herein, and or other similar molecules found in, e.g., GenBank . In other words, the stringency of comparison of the algorithms can be increased so that all known prior art (e.g., those represented by GenBank accession numbers shown herein, or other similar molecules found in, e.g., GenBank) is excluded.

The BLAST algorithm also performs a statistical analysis of the similarity or

15   identity between two sequences (see, e.g., Karlin & Altschul (1993) Proc Natl Acad Sci USA 90:5873-5787). One measure of similarity provided by this algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in

20   a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity or percent sequence

25   similarity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle (1987) J Mol Evol 35:351-360. The method used is similar to the method described by Higgins & Sharp (1989) CABIOS 5:151-153. The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The

30   multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The

final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent

5   sequence identity (or percent sequence similarity) relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, e.g., version 7.0 (Devereaux et al. (1984) Nuc Acids Res 12:387-395).

Another preferred example of an algorithm that is suitable for multiple DNA

10   and amino acid sequence alignments is the CLUSTALW program (Thompson, J. D. et al. (1994) Nuc Acids Res 22:4673-4680). CLUSTALW performs multiple pairwise comparisons between groups of sequences and assembles them into a multiple alignment based on homology. Gap open and Gap extension penalties were 10 and 0.05 respectively. For amino acid alignments, the BLOSUM algorithm can be used as a protein weight matrix

15   (Henikoff and Henikoff (1992) Proc Natl Acad Sci USA 89:10915-10919). Another example of an algorithm suitable for multiple DNA and amino acid sequence alignments is the Jotun Hein method, Hein (1990), from within the MegaLine™ DNASTAR package (MegaLine™ Version 4.03, manufactured by DNASTAR, Inc.) used according to the manufacturer's instructions and default values specified in the program.

20   It will be understood by one of ordinary skill in the art, that the above discussion of search and alignment algorithms also applies to identification and evaluation of polynucleotide sequences, with the substitution of query sequences comprising nucleotide sequences, and where appropriate, selection of nucleic acid databases.

SUBSTRATES AND FORMATS FOR SEQUENCE RECOMBINATION

25   The polynucleotides of the invention and fragments thereof are optionally used as substrates for any of a variety of recombination and recursive sequence recombination reactions, in addition to their use in standard cloning methods as set forth in, e.g., Ausubel, Berger and Sambrook, e.g., to produce additional NCSM polynucleotides or fragments thereof that encode polypeptides and fragments thereof having with desired

30   properties. A variety of such reactions are known, including those developed by the inventors and their co-workers.

DNA recombination is a method for generating and identifying new NCSM molecules, e.g., including those with altered relative binding capacities to either or both of the CD28 and CTLA-4 receptors (as compared to, e.g., hB7-1) and altered functional activities, including, e.g., altered capacities to induce or inhibit T cell activation and/or

5   differentiation, induce or inhibit cytokine production, and/or promote or inhibit anergy and/or tolerance as described herein.

A variety of diversity generating protocols for generating and identifying NCSM molecules having one of more of the properties described herein are available and described in the art. The procedures can be used separately, and/or in combination to

10   produce one or more NCSM variants of a nucleic acid or set of nucleic acids, as well variants of encoded proteins. Individually and collectively, these procedures provide robust, widely applicable ways of generating diversified nucleic acids and sets of nucleic acids (including, e.g., nucleic acid libraries) useful, e.g., for the engineering or rapid evolution of nucleic acids, proteins, pathways, cells and/or organisms with new and/or

15   improved characteristics. While distinctions and classifications are made in the course of the ensuing discussion for clarity, it will be appreciated that the techniques are often not mutually exclusive. Indeed, the various methods can be used singly or in combination, in parallel or in series, to access diverse sequence variants.

The result of any of the diversity generating procedures described herein can

20   be the generation of one or more nucleic acids, which can be selected or screened for nucleic acids with or which confer desirable properties, or that encode proteins with or which confer desirable properties. Following diversification by one or more of the methods herein, or otherwise available to one of skill, any nucleic acids that are generated or produced can be selected for a desired activity or property, e.g. ability to induce or inhibit T

25   cell proliferation or activation, cytokine production, alter binding affinity to one or more of CD28 or CTLA-4 receptors. This can include identifying any activity that can be detected, for example, in an automated or automatable format, by any of the assays in the art and/or the assays of the invention discussed here and/or in the Example section below. A variety of related (or even unrelated) properties can be evaluated, in serial or in parallel, at the

30   discretion of the practitioner.

Descriptions of a variety of diversity generating procedures for generating modified nucleic acid sequences that encode NCSM polypeptides as described herein are found in the following publications and the references cited therein: Soong, N. et al. (2000)

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"Molecular breeding of viruses" <u>Nat Genet</u> 25(4):436-439; Stemmer, et al. (1999) "Molecular breeding of viruses for targeting and other clinical properties" <u>Tumor Targeting</u> 4:1-4; Ness et al. (1999) "DNA Shuffling of subgenomic sequences of subtilisin" <u>Nature Biotechnology</u> 17:893-896; Chang et al. (1999) "Evolution of a cytokine using DNA family

5   shuffling" <u>Nature Biotechnology</u> 17:793-797; Minshull and Stemmer (1999) "Protein evolution by molecular breeding" <u>Current Opinion in Chemical Biology</u> 3:284-290; Christians et al. (1999) "Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling" <u>Nature Biotechnology</u> 17:259-264; Crameri et al. (1998) "DNA shuffling of a family of genes from diverse species accelerates directed evolution"

10   <u>Nature</u> 391:288-291; Crameri et al. (1997) "Molecular evolution of an arsenate detoxification pathway by DNA shuffling," <u>Nature Biotechnology</u> 15:436-438; Zhang et al. (1997) "Directed evolution of an effective fucosidase from a galactosidase by DNA shuffling and screening" <u>Proc. Natl. Acad. Sci. USA</u> 94:4504-4509; Patten et al. (1997) "Applications of DNA Shuffling to Pharmaceuticals and Vaccines" <u>Current Opinion in</u>

15   <u>Biotechnology</u> 8:724-733; Crameri et al. (1996) "Construction and evolution of antibody-phage libraries by DNA shuffling" <u>Nature Medicine</u> 2:100-103; Crameri et al. (1996) "Improved green fluorescent protein by molecular evolution using DNA shuffling" <u>Nature Biotechnology</u> 14:315-319; Gates et al. (1996) "Affinity selective isolation of ligands from peptide libraries through display on a lac repressor 'headpiece dimer'" <u>Journal of Molecular</u>

20   <u>Biology</u> 255:373-386; Stemmer (1996) "Sexual PCR and Assembly PCR" In: <u>The</u> <u>Encyclopedia of Molecular Biology</u>. VCH Publishers, New York. pp.447-457; Crameri and Stemmer (1995) "Combinatorial multiple cassette mutagenesis creates all the permutations of mutant and wildtype cassettes" <u>BioTechniques</u> 18:194-195; Stemmer et al., (1995) "Single-step assembly of a gene and entire plasmid form large numbers of oligodeoxy-

25   ribonucleotides" <u>Gene</u>, 164:49-53; Stemmer (1995) "The Evolution of Molecular Computation" <u>Science</u> 270: 1510; Stemmer (1995) "Searching Sequence Space" <u>Bio/Technology</u> 13:549-553; Stemmer (1994) "Rapid evolution of a protein in vitro by DNA shuffling" <u>Nature</u> 370:389-391; and Stemmer (1994) "DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution." <u>Proc. Natl.</u>

30   <u>Acad. Sci. USA</u> 91:10747-10751.

The term "shuffling" is used herein to indicate recombination between non-identical sequences, in some embodiments shuffling may include crossover via homologous recombination or via non-homologous recombination, such as via cre/lox and/or flp/frt

systems. Shuffling can be carried out by employing a variety of different formats, including for example, *in vitro* and *in vivo* shuffling formats, in silico shuffling formats, shuffling formats that utilize either double-stranded or single-stranded templates, primer based shuffling formats, nucleic acid fragmentation-based shuffling formats, and oligonucleotide-

5   mediated shuffling formats, all of which are based on recombination events between non-identical sequences and are described in more detail or referenced herein below, as well as other similar recombination-based formats.

Mutational methods of generating diversity include, for example, site-directed mutagenesis (Ling et al. (1997) "Approaches to DNA mutagenesis: an overview"

10   Anal Biochem. 254(2): 157-178; Dale et al. (1996) "Oligonucleotide-directed random mutagenesis using the phosphorothioate method" Methods Mol. Biol. 57:369-374; Smith (1985) "In vitro mutagenesis" Ann. Rev. Genet. 19:423-462; Botstein & Shortle (1985) "Strategies and applications of in vitro mutagenesis" Science 229:1193-1201; Carter (1986) "Site-directed mutagenesis" Biochem. J. 237:1-7; and Kunkel (1987) "The efficiency of

15   oligonucleotide directed mutagenesis" in Nucleic Acids & Molecular Biology (Eckstein, F. and Lilley, D.M.J. eds., Springer Verlag, Berlin)); mutagenesis using uracil containing templates (Kunkel (1985) "Rapid and efficient site-specific mutagenesis without phenotypic selection" Proc. Natl. Acad. Sci. USA 82:488-492; Kunkel et al. (1987) "Rapid and efficient site-specific mutagenesis without phenotypic selection" Methods in Enzymol. 154, 367-382;

20   and Bass et al. (1988) "Mutant Trp repressors with new DNA-binding specificities" Science 242:240-245); oligonucleotide-directed mutagenesis (Methods in Enzymol. 100: 468-500 (1983); Methods in Enzymol. 154: 329-350 (1987); Zoller & Smith (1982) "Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any DNA fragment" Nucleic Acids Res.

25   10:6487-6500; Zoller & Smith (1983) "Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors" Methods in Enzymol. 100:468-500; and Zoller & Smith (1987) "Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template" Methods in Enzymol. 154:329-350); phosphorothioate-modified DNA mutagenesis (Taylor et al. (1985) "The use

30   of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA" Nucl. Acids Res. 13: 8749-8764; Taylor et al. (1985) "The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA" Nucl. Acids Res. 13: 8765-8787 (1985); Nakamaye & Eckstein (1986) "Inhibition of

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restriction endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis" Nucl. Acids Res. 14: 9679-9698; Sayers et al. (1988) "Y-T Exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis" Nucl. Acids Res. 16:791-802; and Sayers et al. (1988) "Strand specific cleavage of

5   phosphorothioate-containing DNA by reaction with restriction endonucleases in the presence of ethidium bromide" Nucl. Acids Res. 16: 803-814); mutagenesis using gapped duplex DNA (Kramer et al. (1984) "The gapped duplex DNA approach to oligonucleotide-directed mutation construction" Nucl. Acids Res. 12: 9441-9456; Kramer & Fritz (1987) Methods in Enzymol. "Oligonucleotide-directed construction of mutations via gapped

10   duplex DNA" 154:350-367; Kramer et al. (1988) "Improved enzymatic in vitro reactions in the gapped duplex DNA approach to oligonucleotide-directed construction of mutations" Nucl. Acids Res. 16: 7207; and Fritz et al. (1988) "Oligonucleotide-directed construction of mutations: a gapped duplex DNA procedure without enzymatic reactions in vitro" Nucl. Acids Res. 16: 6987-6999).

15   Additional suitable methods include point mismatch repair (Kramer et al. (1984) "Point Mismatch Repair" Cell 38:879-887), mutagenesis using repair-deficient host strains (Carter et al. (1985) "Improved oligonucleotide site-directed mutagenesis using M13 vectors" Nucl. Acids Res. 13: 4431-4443; and Carter (1987) "Improved oligonucleotide-directed mutagenesis using M13 vectors" Methods in Enzymol. 154: 382-403), deletion

20   mutagenesis (Eghtedarzadeh & Henikoff (1986) "Use of oligonucleotides to generate large deletions" Nucl. Acids Res. 14: 5115), restriction-selection and restriction-purification (Wells et al. (1986) "Importance of hydrogen-bond formation in stabilizing the transition state of subtilisin" Phil. Trans. R. Soc. Lond. A 317: 415-423), mutagenesis by total gene synthesis (Nambiar et al. (1984) "Total synthesis and cloning of a gene coding for the

25   ribonuclease S protein" Science 223: 1299-1301; Sakamar and Khorana (1988) "Total synthesis and expression of a gene for the a-subunit of bovine rod outer segment guanine nucleotide-binding protein (transducin)" Nucl. Acids Res. 14: 6361-6372; Wells et al. (1985) "Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites" Gene 34:315-323; and Grundström et al. (1985) "Oligonucleotide-directed

30   mutagenesis by microscale 'shot-gun' gene synthesis" Nucl. Acids Res. 13: 3305-3316), double-strand break repair (Mandecki (1986) "Oligonucleotide-directed double-strand break repair in plasmids of *Escherichia coli*: a method for site-specific mutagenesis" Proc. Natl. Acad. Sci. USA, 83:7177-7181; and Arnold (1993) "Protein engineering for unusual

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environments" <u>Current Opinion in Biotechnology</u> 4:450-455). Additional details on many of the above methods can be found in <u>Methods in Enzymology</u> Volume 154, which also describes useful controls for trouble-shooting problems with various mutagenesis methods.

Additional details regarding various diversity generating methods can be
5   found in the following U.S. patents, PCT publications and applications, and EPO publications: U.S. Pat. No. 5,605,793 to Stemmer (February 25, 1997), "Methods for In Vitro Recombination;" U.S. Pat. No. 5,811,238 to Stemmer et al. (September 22, 1998) "Methods for Generating Polynucleotides having Desired Characteristics by Iterative Selection and Recombination;" U.S. Pat. No. 5,830,721 to Stemmer et al. (November 3,
10   1998), "DNA Mutagenesis by Random Fragmentation and Reassembly;" U.S. Pat. No. 5,834,252 to Stemmer, et al. (November 10, 1998) "End-Complementary Polymerase Reaction;" U.S. Pat. No. 5,837,458 to Minshull, et al. (November 17, 1998), "Methods and Compositions for Cellular and Metabolic Engineering;" WO 95/22625, Stemmer and Crameri, "Mutagenesis by Random Fragmentation and Reassembly;" WO 96/33207 by
15   Stemmer and Lipschutz "End Complementary Polymerase Chain Reaction;" WO 97/20078 by Stemmer and Crameri "Methods for Generating Polynucleotides having Desired Characteristics by Iterative Selection and Recombination;" WO 97/35966 by Minshull and Stemmer, "Methods and Compositions for Cellular and Metabolic Engineering;" WO 99/41402 by Punnonen et al. "Targeting of Genetic Vaccine Vectors;" WO 99/41383 by
20   Punnonen et al. "Antigen Library Immunization;" WO 99/41369 by Punnonen et al. "Genetic Vaccine Vector Engineering;" WO 99/41368 by Punnonen et al. "Optimization of Immunomodulatory Properties of Genetic Vaccines;" EP 752008 by Stemmer and Crameri, "DNA Mutagenesis by Random Fragmentation and Reassembly;" EP 0932670 by Stemmer "Evolving Cellular DNA Uptake by Recursive Sequence Recombination;" WO 99/23107 by
25   Stemmer et al., "Modification of Virus Tropism and Host Range by Viral Genome Shuffling;" WO 99/21979 by Apt et al., "Human Papillomavirus Vectors;" WO 98/31837 by del Cardayre et al. "Evolution of Whole Cells and Organisms by Recursive Sequence Recombination;" WO 98/27230 by Patten and Stemmer, "Methods and Compositions for Polypeptide Engineering;" WO 98/27230 by Stemmer et al., "Methods for Optimization of
30   Gene Therapy by Recursive Sequence Shuffling and Selection," WO 00/00632, "Methods for Generating Highly Diverse Libraries," WO 00/09679, "Methods for Obtaining in Vitro Recombined Polynucleotide Sequence Banks and Resulting Sequences," WO 98/42832 by Arnold et al., "Recombination of Polynucleotide Sequences Using Random or Defined

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Primers," WO 99/29902 by Arnold et al., "Method for Creating Polynucleotide and

Polypeptide Sequences," WO 98/41653 by Vind, "An in Vitro Method for Construction of a

DNA Library," WO 98/41622 by Borchert et al., "Method for Constructing a Library Using

DNA Shuffling," and WO 98/42727 by Pati and Zarling, "Sequence Alterations using

5   Homologous Recombination;" WO 00/18906 by Patten et al., "Shuffling of Codon-Altered

Genes;" WO 00/04190 by del Cardayre et al. "Evolution of Whole Cells and Organisms by

Recursive Recombination;" WO 00/42561 by Crameri et al., "Oligonucleotide Mediated

Nucleic Acid Recombination;" WO 00/42559 by Selifonov and Stemmer "Methods of

Populating Data Structures for Use in Evolutionary Simulations;" WO 00/42560 by

10   Selifonov et al., "Methods for Making Character Strings, Polynucleotides & Polypeptides

Having Desired Characteristics;" PCT/US00/26708 by Welch et al., "Use of Codon-Varied

Oligonucleotide Synthesis for Synthetic Shuffling;" and PCT/US01/06775 "Single-Stranded

Nucleic Acid Template-Mediated Recombination and Nucleic Acid Fragment Isolation" by

Affholter.

15   Several different general classes of sequence modification methods, such as

mutation, recombination, etc. are applicable to the present invention and set forth, e.g., in

the references above and below. That is, nucleic acids encoding CD28BP or CTLA-4BP

polypeptides can be diversified by any of the methods described herein, *e.g.,* including

various mutation and recombination methods, individually or in combination, to generate

20   nucleic acids with a desired activity or property, including, e.g., those described herein, such

as an ability to enhance an immune response, such as by inducing T cell activation or

proliferation, an ability to down-regulate or inhibit an immune response, such as by

inhibiting T cell activation or proliferation, an ability to preferentially bind and/or signal

through either or both CD28 and CTLA-4 receptors.

25   The following exemplify some of the different types of preferred formats for

diversity generation in the context of the present invention, including, e.g., certain

recombination based diversity generation formats.

               Nucleic acids can be recombined in vitro by any of a variety of techniques

discussed in the references above, including e.g., DNAse digestion of nucleic acids to be

30   recombined followed by ligation and/or PCR reassembly of the nucleic acids. For example,

sexual PCR mutagenesis can be used in which random (or pseudo random, or even non-

random) fragmentation of the DNA molecule is followed by recombination, based on

sequence similarity, between DNA molecules with different but related DNA sequences, in

vitro, followed by fixation of the crossover by extension in a polymerase chain reaction. This process and many process variants is described in several of the references above, e.g., in Stemmer (1994) Proc. Natl. Acad. Sci. USA 91:10747-10751. Thus, nucleic acids encoding B7-1 polypeptides and subsequences thereof can be recombined in vitro to

5   generate nucleic acids encoding modified or recombinant B7-1 polypeptides, NCSM polypeptides, CD28BP polypeptides, CTLA-4BP polypeptides, or subsequences thereof, each of which has one or more desired properties, including those described herein.

Similarly, nucleic acids can be recursively recombined in vivo, e.g., by allowing recombination to occur between nucleic acids in cells. Many such in vivo

10   recombination formats are set forth in the references noted above. Such formats optionally provide direct recombination between nucleic acids of interest, or provide recombination between vectors, viruses, plasmids, etc., comprising the nucleic acids of interest, as well as other formats. Details regarding such procedures are found in the references noted above. Thus, nucleic acids encoding B7-1 polypeptides and subsequences thereof can be

15   recombined *in vivo* prior to, or in concert with screening and/or selection procedures to identify modified or recombinant B7-1 polypeptides, NCSM polypeptides, CD28BP polypeptides, CTLA-4BP polypeptides, or subsequences thereof, each of which has one or more desired properties, including those described herein. Whole genome recombination methods can also be used in which whole genomes of cells or other organisms are

20   recombined, optionally including spiking of the genomic recombination mixtures with desired library components (e.g., genes corresponding to the pathways of the present invention). These methods have many applications, including those in which the identity of a target gene is not known. Details on such methods are found, e.g., in WO 98/31837 by del Cardayre et al. "Evolution of Whole Cells and Organisms by Recursive Sequence

25   Recombination;" and in, e.g., PCT/US99/15972 by del Cardayre et al., also entitled "Evolution of Whole Cells and Organisms by Recursive Sequence Recombination."

Synthetic recombination methods can also be used, in which oligonucleotides corresponding to targets of interest (e.g., B7 polypeptides) are synthesized and reassembled in PCR or ligation reactions which include oligonucleotides which

30   correspond to more than one parental nucleic acid, thereby generating new recombined nucleic acids. Oligonucleotides can be made by standard nucleotide addition methods, or can be made, e.g., by tri-nucleotide synthetic approaches. Details regarding such approaches are found in the references noted above, including, e.g., WO 00/42561 by

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Crameri et al., "Oligonucleotide Mediated Nucleic Acid Recombination;"
PCT/US00/26708 by Welch et al., "Use of Codon-Varied Oligonucleotide Synthesis for
Synthetic Shuffling;" WO 00/42560 by Selifonov et al., "Methods for Making Character
Strings, Polynucleotides and Polypeptides Having Desired Characteristics;" and WO

5   00/42559 by Selifonov and Stemmer "Methods of Populating Data Structures for Use in
Evolutionary Simulations."

In silico methods of recombination can be effected in which genetic
algorithms are used in a computer to recombine sequence strings which correspond to
homologous (or even non-homologous) nucleic acids. The resulting recombined sequence

10   strings are optionally converted into nucleic acids by synthesis of nucleic acids which
correspond to the recombined sequences, e.g., in concert with oligonucleotide synthesis/
gene reassembly techniques. This approach can generate random, partially random or
designed variants. Many details regarding in silico recombination, including the use of
genetic algorithms, genetic operators and the like in computer systems, combined with

15   generation of corresponding nucleic acids (and/or proteins), as well as combinations of
designed nucleic acids and/or proteins (e.g., based on cross-over site selection) as well as
designed, pseudo-random or random recombination methods are described in WO 00/42560
by Selifonov et al., "Methods for Making Character Strings, Polynucleotides and
Polypeptides Having Desired Characteristics" and WO 00/42559 by Selifonov and Stemmer

20   "Methods of Populating Data Structures for Use in Evolutionary Simulations." Extensive
details regarding in silico recombination methods are found in these applications. This
methodology is generally applicable to the present invention in providing for recombination
of the character strings corresponding to nucleic acids encoding co-stimulatory B7
molecules in silico and/or the generation of corresponding nucleic acids or proteins.

25   Many methods of accessing natural diversity, e.g., by hybridization of
diverse nucleic acids or nucleic acid fragments to single-stranded templates, followed by
polymerization and/or ligation to regenerate full-length sequences, optionally followed by
degradation of the templates and recovery of the resulting modified nucleic acids can be
similarly used. In one method employing a single-stranded template, the fragment

30   population derived from the genomic library(ies) is annealed with partial, or, often
approximately full length ssDNA or RNA corresponding to the opposite strand. Assembly
of complex chimeric genes from this population is then mediated by nuclease-base removal
of non-hybridizing fragment ends, polymerization to fill gaps between such fragments and

subsequent single stranded ligation. The parental polynucleotide strand can be removed by digestion (e.g., if RNA or uracil-containing), magnetic separation under denaturing conditions (if labeled in a manner conducive to such separation) and other available separation/purification methods. Alternatively, the parental strand is optionally co-purified

5 with the chimeric strands and removed during subsequent screening and processing steps. Additional details regarding this approach are found, e.g., in "Single-Stranded Nucleic Acid Template-Mediated Recombination and Nucleic Acid Fragment Isolation" by Affholter, PCT/US01/06775.

In another approach, single-stranded molecules are converted to double-

10 stranded DNA (dsDNA) and the dsDNA molecules are bound to a solid support by ligand-mediated binding. After separation of unbound DNA, the selected DNA molecules are released from the support and introduced into a suitable host cell to generate a library enriched sequences which hybridize to the probe. A library produced in this manner provides a desirable substrate for further diversification using any of the procedures

15 described herein.

Any of the preceding general recombination formats can be practiced in a reiterative fashion (e.g., one or more cycles of mutation/recombination or other diversity generation methods, optionally followed by one or more selection methods) to generate a more diverse set of recombinant nucleic acids.

20 Mutagenesis employing polynucleotide chain termination methods have also been proposed (*see* e.g., U.S. Patent No. 5,965,408, "Method of DNA reassembly by interrupting synthesis" to Short, and the references above), and can be applied to the present invention. In this approach, double stranded DNAs corresponding to one or more genes sharing regions of sequence similarity are combined and denatured, in the presence or

25 absence of primers specific for the gene. The single stranded polynucleotides are then annealed and incubated in the presence of a polymerase and a chain terminating reagent (e.g., ultraviolet, gamma or X-ray irradiation; ethidium bromide or other intercalators; DNA binding proteins, such as single strand binding proteins, transcription activating factors, or histones; polycyclic aromatic hydrocarbons; trivalent chromium or a trivalent chromium

30 salt; or abbreviated polymerization mediated by rapid thermocycling; and the like), resulting in the production of partial duplex molecules. The partial duplex molecules, e.g., containing partially extended chains, are then denatured and reannealed in subsequent rounds of replication or partial replication resulting in polynucleotides which share varying

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degrees of sequence similarity and which are diversified with respect to the starting population of DNA molecules. Optionally, the products, or partial pools of the products, can be amplified at one or more stages in the process. Polynucleotides produced by a chain termination method, such as described above, are suitable substrates for any other described

5   recombination format.

Diversity also can be generated in nucleic acids or populations of nucleic acids using a recombinational procedure termed "incremental truncation for the creation of hybrid enzymes" ("ITCHY") described in Ostermeier et al. (1999) "A combinatorial approach to hybrid enzymes independent of DNA homology" Nature Biotech 17:1205.

10   This approach can be used to generate an initial a library of variants which can optionally serve as a substrate for one or more in vitro or in vivo recombination methods. See, also, Ostermeier et al. (1999) "Combinatorial Protein Engineering by Incremental Truncation," Proc. Natl. Acad. Sci. USA, 96: 3562-67; Ostermeier et al. (1999), "Incremental Truncation as a Strategy in the Engineering of Novel Biocatalysts," Biological and Medicinal

15   Chemistry, 7: 2139-44.

Mutational methods which result in the alteration of individual nucleotides or groups of contiguous or non-contiguous nucleotides can be favorably employed to introduce nucleotide diversity. For example, mutagenesis procedures resulting in changes of one or more nucleotides can be used to generate any number of nucleic acids encoding

20   polypeptides of the present invention. Many mutagenesis methods are found in the above-cited references; additional details regarding mutagenesis methods can be found in following, which can also be applied to the present invention.

For example, error-prone PCR can be used to generate nucleic acid variants. Using this technique, PCR is performed under conditions where the copying fidelity of the

25   DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Examples of such techniques are found in the references above and, e.g., in Leung et al. (1989) Technique 1:11-15 and Caldwell et al. (1992) PCR Methods Applic. 2:28-33. Similarly, assembly PCR can be used, in a process which involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of

30   different PCR reactions can occur in parallel in the same reaction mixture, with the products of one reaction priming the products of another reaction.

Oligonucleotide directed mutagenesis can be used to introduce site-specific mutations in a nucleic acid sequence of interest. Examples of such techniques are found in

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the references above and, e.g., in Reidhaar-Olson et al. (1988) _Science_, 241:53-57. Similarly, cassette mutagenesis can be used in a process that replaces a small region of a double stranded DNA molecule with a synthetic oligonucleotide cassette that differs from the native sequence. The oligonucleotide can contain, e.g., completely and/or partially randomized native sequence(s).

Recursive ensemble mutagenesis is a process in which an algorithm for protein mutagenesis is used to produce diverse populations of phenotypically related mutants, members of which differ in amino acid sequence. This method uses a feedback mechanism to monitor successive rounds of combinatorial cassette mutagenesis. Examples of this approach are found in Arkin & Youvan (1992) _Proc. Natl. Acad. Sci. USA_ 89:7811-7815.

Exponential ensemble mutagenesis can be used for generating combinatorial libraries with a high percentage of unique and functional mutants. Small groups of residues in a sequence of interest are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins. Examples of such procedures are in Delegrave & Youvan (1993) _Biotechnology Research_ 11:1548-1552.

In vivo mutagenesis can be used to generate random mutations in any cloned DNA of interest by propagating the DNA, e.g., in a strain of _E. coli_ that carries mutations in one or more of the DNA repair pathways. These "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA. Such procedures are described in the references noted above.

Other procedures for introducing diversity into a genome, e.g. a bacterial, fungal, animal or plant genome can be used in conjunction with the above described and/or referenced methods. For example, in addition to the methods above, techniques have been proposed which produce nucleic acid multimers suitable for transformation into a variety of species (_see,_ e.g., Schellenberger U.S. Patent No. 5,756,316 and the references above). Transformation of a suitable host with such multimers, consisting of genes that are divergent with respect to one another, (e.g., derived from natural diversity or through application of site directed mutagenesis, error prone PCR, passage through mutagenic bacterial strains, and the like), provides a source of nucleic acid diversity for DNA diversification, e.g., by an in vivo recombination process as indicated above.

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Alternatively, a multiplicity of monomeric polynucleotides sharing regions of partial sequence similarity can be transformed into a host species and recombined in vivo by the host cell. Subsequent rounds of cell division can be used to generate libraries, members of which, include a single, homogenous population, or pool of monomeric

5　polynucleotides. Alternatively, the monomeric nucleic acid can be recovered by standard techniques, e.g., PCR and/or cloning, and recombined in any of the recombination formats, including recursive recombination formats, described above.

Methods for generating multispecies expression libraries have been described (in addition to the reference noted above, *see*, e.g., Peterson et al. (1998) U.S. Pat.

10　No. 5,783,431 "METHODS FOR GENERATING AND SCREENING NOVEL METABOLIC PATHWAYS," and Thompson, et al. (1998) U.S. Pat. No. 5,824,485 METHODS FOR GENERATING AND SCREENING NOVEL METABOLIC PATHWAYS) and their use to identify protein activities of interest has been proposed (In addition to the references noted above, see Short (1999) U.S. Pat. No. 5,958,672

15　"PROTEIN ACTIVITY SCREENING OF CLONES HAVING DNA FROM UNCULTIVATED MICROORGANISMS"). Multispecies expression libraries include, in general, libraries comprising cDNA or genomic sequences from a plurality of species or strains, operably linked to appropriate regulatory sequences, in an expression cassette. The cDNA and/or genomic sequences are optionally randomly ligated to further enhance

20　diversity. The vector can be a shuttle vector suitable for transformation and expression in more than one species of host organism, e.g., bacterial species, eukaryotic cells. In some cases, the library is biased by preselecting sequences which encode a protein of interest, or which hybridize to a nucleic acid of interest. Any such libraries can be provided as substrates for any of the methods herein described.

25　The above-described procedures have been largely directed to increasing nucleic acid and/ or encoded protein diversity. However, in many cases, not all of the diversity is useful, e.g., functional, and contributes merely to increasing the background of variants that must be screened or selected to identify the few favorable variants. In some applications, it is desirable to preselect or prescreen libraries (e.g., an amplified library, a

30　genomic library, a cDNA library, a normalized library, etc.) or other substrate nucleic acids prior to diversification, e.g., by recombination-based mutagenesis procedures, or to otherwise bias the substrates towards nucleic acids that encode functional products. For example, in the case of antibody engineering, it is possible to bias the diversity generating

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process toward antibodies with functional antigen binding sites by taking advantage of in vivo recombination events prior to manipulation by any of the described methods. For example, recombined CDRs derived from B cell cDNA libraries can be amplified and assembled into framework regions (e.g., Jirholt et al. (1998) "Exploiting sequence space:

5   shuffling in vivo formed complementarity determining regions into a master framework" Gene 215: 471) prior to diversifying according to any of the methods described herein.

Libraries can be biased towards nucleic acids which encode proteins with desirable enzyme activities. For example, after identifying a clone from a library which exhibits a specified activity, the clone can be mutagenized using any known method for

10   introducing DNA alterations. A library comprising the mutagenized homologues is then screened for a desired activity, which can be the same as or different from the initially specified activity. An example of such a procedure is proposed in Short (1999) U.S. Patent No. 5,939,250 for "PRODUCTION OF ENZYMES HAVING DESIRED ACTIVITIES BY MUTAGENESIS." Desired activities can be identified by any method known in the art.

15   For example, WO 99/10539 proposes that gene libraries can be screened by combining extracts from the gene library with components obtained from metabolically rich cells and identifying combinations which exhibit the desired activity. It has also been proposed (e.g., WO 98/58085) that clones with desired activities can be identified by inserting bioactive substrates into samples of the library, and detecting bioactive fluorescence corresponding to

20   the product of a desired NCSM activity as described herein using a fluorescent analyzer, e.g., a flow cytometry device, a CCD, a fluorometer, or a spectrophotometer.

Libraries can also be biased towards nucleic acids which have specified characteristics, e.g., hybridization to a selected nucleic acid probe. For example, application WO 99/10539 proposes that polynucleotides encoding a desired activity (e.g., an enzymatic

25   activity, for example: a lipase, an esterase, a protease, a glycosidase, a glycosyl transferase, a phosphatase, a kinase, an oxygenase, a peroxidase, a hydrolase, a hydratase, a nitrilase, a transaminase, an amidase or an acylase) can be identified from among genomic DNA sequences in the following manner. Single stranded DNA molecules from a population of genomic DNA are hybridized to a ligand-conjugated probe. The genomic DNA can be

30   derived from either a cultivated or uncultivated microorganism, or from an environmental sample. Alternatively, the genomic DNA can be derived from a multicellular organism, or a tissue derived therefrom. Second strand synthesis can be conducted directly from the hybridization probe used in the capture, with or without prior release from the capture

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medium or by a wide variety of other strategies known in the art. Alternatively, the isolated single-stranded genomic DNA population can be fragmented without further cloning and used directly in, e.g., a recombination-based approach, that employs a single-stranded template, as described above.

5   "Non-Stochastic" methods of generating nucleic acids and polypeptides are alleged in Short "Non-Stochastic Generation of Genetic Vaccines and Enzymes" WO 00/46344. These methods, including proposed non-stochastic polynucleotide reassembly and site-saturation mutagenesis methods be applied to the present invention as well. Random or semi-random mutagenesis using doped or degenerate oligonucleotides is also

10   described in, e.g., Arkin and Youvan (1992) "Optimizing nucleotide mixtures to encode specific subsets of amino acids for semi-random mutagenesis" *Biotechnology* 10:297-300; Reidhaar-Olson et al. (1991) "Random mutagenesis of protein sequences using oligonucleotide cassettes" *Methods Enzymol.* 208:564-86; Lim and Sauer (1991) "The role of internal packing interactions in determining the structure and stability of a protein" *J.*

15   *Mol. Biol.* 219:359-76; Breyer and Sauer (1989) "Mutational analysis of the fine specificity of binding of monoclonal antibody 51F to lambda repressor" *J. Biol. Chem.* 264:13355-60); and "Walk-Through Mutagenesis" (Crea, R; US Patents 5,830,650 and 5,798,208, and EP Patent 0527809 B1.

        It will readily be appreciated that any of the above described techniques

20   suitable for enriching a library prior to diversification can also be used to screen the products, or libraries of products, produced by the diversity generating methods.

        Kits for mutagenesis, library construction and other diversity generation methods are also commercially available. For example, kits are available from, e.g., Stratagene (e.g., QuickChange™ site-directed mutagenesis kit; and Chameleon™ double-

25   stranded, site-directed mutagenesis kit), Bio/Can Scientific, Bio-Rad (e.g., using the Kunkel method described above), Boehringer Mannheim Corp., Clonetech Laboratories, DNA Technologies, Epicentre Technologies (e.g., 5 prime 3 prime kit); Genpak Inc, Lemargo Inc, Life Technologies (Gibco BRL), New England Biolabs, Pharmacia Biotech, Promega Corp., Quantum Biotechnologies, Amersham International plc (e.g., using the Eckstein

30   method above), and Anglian Biotechnology Ltd (e.g., using the Carter/Winter method above).

        The above references provide many mutational formats, including recombination, recursive recombination, recursive mutation and combinations or

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recombination with other forms of mutagenesis, as well as many modifications of these formats. Regardless of the diversity generation format that is used, the nucleic acids of the invention can be recombined (with each other, or with related (or even unrelated) sequences) to produce a diverse set of recombinant nucleic acids, including, e.g., sets of

5   homologous nucleic acids, as well as corresponding polypeptides.

A recombinant nucleic acid produced by recombining one or more polynucleotide sequences of the invention with one or more additional nucleic acids using any of the above-described formats alone or in combination also forms a part of the invention. The one or more additional nucleic acids may include another polynucleotide of

10   the invention; optionally, alternatively, or in addition, the one or more additional nucleic acids can include, e.g., a nucleic acid encoding a naturally-occurring B7-1, co-stimulatory homologue or a subsequence thereof, or any homologous B7-1, co-stimulatory sequence or subsequence thereof (e.g., as found in GenBank or other available literature), or, e.g., any other homologous or non-homologous nucleic acid or fragments thereof (certain

15   recombination formats noted above, notably those performed synthetically or in silico, do not require homology for recombination).

POLYPEPTIDES OF THE INVENTION

The invention provides isolated or recombinant NCSM polypeptides, fragments thereof, and homologues, variants and derivatives thereof, collectively referred to

20   herein as "NCSM polypeptides" or "NCSM polypeptide" unless otherwise specifically noted. The term "NCSM polypeptide" is intended throughout to include amino acid fragments, homologues, derivatives, variants of the polypeptide and protein sequences specifically disclosed herein unless otherwise noted. Polypeptide variants include those with conservative amino acid substations ("conservatively substituted variations") as

25   described above. Also included in this invention are fusion proteins comprising NCSM polypeptides and proteins, chimeric NCSM polypeptides, comprising one or more fragments from one or more NCSM polypeptides set forth herein.

As discussed above, the invention provides CD28BP polypeptides and CTLA-4BP polypeptides and fragments of either thereof that bind either or both of CD28 or

30   CTLA-4 receptor. In some embodiments, a CD28BP polypeptide of the invention (including fragments thereof, such as soluble ECDs and ECD fusion proteins, cytoplasmic domains, transmembrane domains, and/or signal peptides, and fusion proteins thereof) has a

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binding affinity for CD28 that is about equal to or greater than that of hB7-1 for CD28 (which is about 4×10^{-6} M) and/or a binding affinity for CTLA-4 that is about equal to or less than about that of hB7-1 for CTLA-4 (i.e., about $0.2 - 0.4 \times 10^{-6}$ M). In other embodiments, a CD28BP of the invention has a CD28/CTLA-4 binding affinity ratio that is

5   about equal to or greater than that of hB7-1. In some such embodiments, a ratio of specific binding affinities CD28/CTLA-4 for a CD28BP is at least about $0.5 - 1 \times 10^{-1}$.

In other embodiments, a CTLA-4BP polypeptide of the invention (including fragments thereof, such as soluble ECDs and ECD fusion proteins, cytoplasmic domains, transmembrane domains, and/or signal peptides, and fusion proteins thereof) has a CTLA-

10   4/CD28 binding affinity ratio that is about equal to or greater than that of hB7-1. In some embodiments, a CTLA-4BP polypeptide of the invention has a binding affinity for CTLA-4 that is about equal to or greater than that of hB7-1 for CTLA-4 (i.e., about 4×10^{-6} M) and/or a binding affinity for CD28 that is about equal to or less than that of hB7-1 for CD28 (which ranges from about 0.2×10^{-6} M to about 0.4×10^{-6} M). In other embodiments, a

15   CTLA-4BP has a binding affinity for CTLA-4 and CD28 that is less than the binding affinity of hB7-1 for either receptor; however, the ratio of these binding affinities ratio (CTLA-4/CD28) is still at least equal to or greater than that of hB7-1. In other embodiments, for a CTLA-4BP, a ratio of specific binding affinities CTLA-4/ CD28 is at least about 10. Also included are nucleic acid sequences (e.g., DNA and RNA) that encode

20   all aforementioned NCSM polypeptides and fragments thereof having one or more properties outlined above, vectors comprising such nucleic acid sequences, cells transformed with such vectors.

CD28BP Polypeptides

In one aspect, the invention provides an isolated or recombinant CD28BP

25   polypeptides comprising an extracellular domain (ECD) sequence, wherein the ECD sequence has at least about 65%, 70% 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% or more amino acid sequence identity to an extracellular domain amino acid sequence of at least one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, and is not a naturally-occurring extracellular domain amino acid sequence, and

30   wherein said polypeptide has a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1.

For some such polypeptides, the polypeptide comprises an extracellular domain amino acid sequence or the full-length amino acid sequence of any one of SEQ ID

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NOS:48-68, 174-182, 184-221, 283-285, and 290-293. Some such polypeptides comprise an extracellular domain amino acid sequence of any one of SEQ ID NOS:48-68 and 174-209. For some such polypeptides, the polypeptide comprises an extracellular domain amino acid sequence encoded by a coding polynucleotide sequence that is selected from the group

5   of: (a) an ECD coding sequence of a polynucleotide sequence selected from any of SEQ ID NOS:1-21 and 95-142; (b) an polynucleotide sequence that encodes the ECD of a polypeptide selected from any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293; and (c) a polynucleotide sequence which, but for codon degeneracy, hybridizes under stringent conditions over substantially the entire length of a polynucleotide sequence (a) or (b).

10   Some such CD28BP polypeptides described above have an equal or enhanced binding affinity for CD28 as compared to a binding affinity of a WT co-stimulatory molecule for CD28. Some such polypeptides have a CD28/CTLA-4 binding affinity ratio at least equal to or greater than the CD28/CTLA-4 binding affinity ratio of hB7-1. In one aspect, some such polypeptides have a decreased or a lowered binding

15   affinity for CTLA-4 as compared to a binding affinity of a wild type co-stimulatory molecule for CTLA-4. Some such CD28BP polypeptides may induce T-cell proliferation or T-cell activation or both T-cell proliferation and T-cell activation, such as, e.g., in association with co-stimulation of T cell receptor/CD3 (by, e.g., an antigen or anti-CD3 antibody). The induced T-cell proliferative response may be about equal to or greater than

20   that of human B7-1 for some such polypeptides. In another aspect, some such polypeptides described above modulate T-cell activation, but do not induce proliferation of purified T-cells activated by soluble anti-CD3 mAbs.

In another aspect, the invention provides isolated or recombinant CD28BP polypeptides that comprise a non-naturally-occurring amino acid sequence encoded by a

25   nucleic acid comprising a polynucleotide sequence selected from the group of: (a) a polynucleotide sequence selected from SEQ ID NOS:1-21 and 95-142, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which, but for degeneracy

30   of the genetic code, hybridizes under highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b); (d) a polynucleotide sequence comprising all or a fragment of (a), (b), or (c), wherein the fragment encodes a polypeptide having a CD28/CTLA-4 binding affinity ratio about equal to or greater than the

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CD28/CTLA-4 binding affinity ratio of human B7-1; (e) a polynucleotide sequence encoding a polypeptide, the polypeptide comprising an amino acid sequence which is substantially identical over at least about 150 contiguous amino acid residues of any one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293; and (f) a polynucleotide sequence

5 encoding a polypeptide that has a CD28/CTLA-4 binding affinity ratio equal to, about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1, which polynucleotide sequence has at least about 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more identity to at least one polynucleotide sequence of (a), (b), (c), or (d). In one aspect, such CD28BP polypeptides comprise the

10 full-length amino acid sequence of any one of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293.

The above-described polypeptides have a CD28/CTLA-4 binding affinity ratio about equal to, equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1. Some such polypeptides induce a T-cell proliferation in association with TCR

15 stimulation; the response may be about equal to or greater than that of human B7-1.

In yet another embodiment, the invention provides isolated or recombinant polypeptides comprising an amino acid sequence according to the formula:

MGHTM-X6-W-X8-SLPPK-X14-PCL-X18-X19-X20-QLLVLT-X27-LFYFCSGITPKSVTKRVKETVMLSCDY-X55-TSTE-X60-LTSLRIYW-X69-

20 KDSKMVLAILPGKVQVWPEYKNRTITDMNDN-X101-RIVI-X106-ALR-X110-SD-X113-GTYTCV-X120-QKP-X124-LKGAYKLEHL-X135-SVRLMIRADFPVP-X149-X150-X151-DLGNPSPNIRRLICS-X167-X168-X169-GFPRPHL-X177-WLENGEELNATNTT-X192-SQDP-X197-T-X199-LYMISSEL-X208-FNVTNN-X215-SI-X218-CLIKYGEL-X227-VSQIFPWSKPKQEPPIDQLPF-X249-VIIPVSGALVL-X261-

25 A-X263-VLY-X267-X268-ACRH-X273-ARWKRTRRNEETVGTE RLSPIYLGSAQSSG (SEQ ID NO:284), or a subsequence thereof comprising the extracellular domain, wherein position X6 is Lys or Glu; position X8 is Arg or Gly; position X14 is Arg or Cys; position X18 is Trp or Arg; position X19 is Pro or Leu; position X20 is Ser or Pro; position X27 is Asp or Gly; position X55 is Asn or Ser; position X60 is Glu or Lys; position X69 is Gln or

30 Arg; position X101 is Pro or Leu; position X106 is Leu or Gln; position X110 is Pro or Leu; position X113 is Lys or Ser; position X120 is Val or Ile; position X124 is Val or Asp; position X135 is Thr or Ala; position X149 is Thr, Ser, or del; position X150 is Ile or del; position X151 is Asn or Thr; position X167 is Thr or del; position X169 is Ser or del;

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position X169 is Gly or del; position X177 is Cys or Tyr; position X192 is Val or Leu; position X197 is Gly or Glu; position X199 is Glu or Lys; position X208 is Gly or Asp; position X215 is His or Arg; position X218 is Ala or Val; position X227 is Ser or Leu; position X249 is Trp, Leu, or Arg; position X261 is Ala or Thr; position X263 is Val, Ala, or Ile; position X267 is Arg or Cys; position X268 is Pro or Leu; and position X273 is Gly or Val. Some such polypeptides have one or more of the properties of CD28 polypeptides described herein, including an ability to enhance an immune response, induce a T cell activation or proliferation response, exhibit a CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1, and/or alter cytokine production. For some such polypeptides, the induced T cell response is about equal to or greater than that of hB7-1. In one embodiment, some such polypeptides comprise an extracellular domain amino acid sequence of any one of SEQ ID NOS:51-56, 58, 61, 66, 67, 174-179, 181, 185-187, 189, 192-194, 197, 199, 202, 205, 208, 215, 217, 220, and 285.

In another embodiment, some such polypeptides comprise two, three, four, five, six, eight, ten, or more of: Lys at position X6; Arg at position X8; Arg at position X14; Trp at position X18; Pro at position X19; Ser at position X20; Asp at position X27; Asn at position X55; Leu at position X106; Pro at position X110; Lys at position X113; Val at position X120; Val at position X124; Thr at position X135; Asn at position X151; Cys at position X177; Val at position X192; Gly at position X197; Glu at position X199; Gly at position X208; His at position X215; Ala at position X218; Trp at position X249; Ala at position X261; Val at position X263; Arg at position X267; Pro at position X268; and Gly at position X273. In a preferred embodiment, some such polypeptides comprise two, three, four, five, six, eight, ten, or more of: Arg at position X8; Arg at position X14; Trp at position X18; Pro at position X19; Ser at position X20; Pro at position X110; Val at position X120; Val at position X124; Cys at position X177; Val at position X192; Gly at position X197; Glu at position X199; Gly at position X208; His at position X215; Ala at position X218; Trp at position X249; Ala at position X261; and Val at position X263. In yet another preferred embodiment, some such polypeptides comprise the extracellular domain amino acid sequence of SEQ ID NO:66 or SEQ ID NO:285. In yet another preferred embodiment, some such polypeptides comprise the sequence of SEQ ID NO:66 or SEQ ID NO:285.

In another aspect, the invention provides isolated or recombinant CD28BP polypeptides comprising a subsequence of an amino acid sequence set forth in any of SEQ

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ID NOS:48-68, 174-182, 184-221, 283-285, and 290-293, wherein the subsequence is the extracellular domain of said amino acid sequence.

The invention also provides isolated or recombinant polypeptides comprising an amino acid sequence according to the formula:

MGHTMKWGSLPPKRPCLWLSQLLVLTGLFYFCSGITPK SVTKRVKETVM-X50-SCDY-X55-X56-STEELTSLRIYWQKDSKMVL AILPGKVQVWPEYKNRTITD MNDNPRIVILALRLSD-X113-GTYTCV-X120-QK-X123-X124-X125-X126-G-X128-X129-X130-X131-EHL-X135-SV-X138-L-X140-IRADFPVPSITDIGHPAPNVK RIRCSASG-X170-FPEPRLAWMEDGEEL NAVNTTV-X193-X194-X195-LDTELYSVSSELD-X209-N-X211-TNNHSIVCLIKYGELSVSQIFPWSKPK QEPPIDQLPFWVI-X252-X253-VSGALVLTAVVLYCLACRHVAR (SEQ ID NO:290), or subsequence thereof comprising the extracellular domain, wherein position X50 is Leu or Pro; position X55 is Asn or Ser; position X56 is Ala or Thr; position X113 is Ser or Lys; position X120 is Ile or Val; position X123 is Pro or deleted; position X124 is Val, Asn, or Asp; position X125 is Leu or Glu; position X126 is Lys or Asn; position X128 is Ala or Ser; position X129 is Tyr or Phe; position X130 is Lys or Arg; position X131 is Leu or Arg; position X135 is Ala or Thr; position X138 is Arg or Thr; position X140 is Met or Ser; position X170 is Asp or Gly; position X193 is Asp or is deleted; position X194 is Gln or is deleted; position X195 is Asp or is deleted; position X211 is Val or Ala; position X252 is Ile or Val; and position X253 is Leu or Pro. Some such polypeptides have at least one of the properties of CD28 polypeptides described herein, including an ability to enhance an immune response, induce T cell activation or proliferation, exhibit a CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1, and/or alter cytokine production. For some such polypeptides, the induced T cell response is about equal to or greater than that of hB7-1.

In a preferred embodiment, some such CD28BP polypeptides comprise two, three, four, five, six, eight, ten, or more of: Leu at position X50; Asn at position X55; Ala at position X56; Ser at position X113; Ile at position X120; Pro at position X123; Val at position X124; Leu at position X125; Lys at position X126; Ala at position X128; Tyr at position X129; Lys at position X130; Leu at position X131; Ala at position X135; Arg at position X138; Met at position X140; Asp at position X170; Asp at position X193; Asp at position X194; Asp at position X195; Val at position X211; Ile at position X252; and Leu at position X253. In yet another preferred embodiment, some such polypeptides comprise a

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sequence of any one of SEQ ID NOS:59, 62, 180, 184, 188, 195, 196, 200, 201, 204, 211, 213, 219, and 291.

In another aspect, the invention provides isolated or recombinant polypeptides comprising an amino acid sequence according to the formula:

MGHTMKWG-X9-LPPKRPCLWLSQLLVLTGLFYFCSG-X35-TPKSVTKRV KETVMLSCDY-X55-TSTEELTSLRIYWQKDSKMVLAILPGKVQVW PEYKNRTITDMNDNPRIVILALR-X110-SDSGTYTCVIQKP-X124-LKGAYKLEHL-X135-SVRLMIRADFPVPTINDLGNPSPNIRRLICSTSGGFPRPHLYWLENG-X183-ELNATNTT-X192-SQDPETKLYMISSELDFN-X211-TSN-X215-X216-X217-LCLVKYGDLTVSQ-X231-FYWQESKPTPSANQHLTWTIIIPVSAFGISVIIAVI LTCLTCRNAAIRRQRRENEV-X288-M-X290-SCSQSP (SEQ ID NO:292), or a subsequence thereof comprising the extracellular domain, wherein position X9 is Thr or Ser; position X35 is Ile or Thr; position X55 is Asn or Ser; position X110 is Leu or Pro; position X124 is Asp or Val; position X135 is Thr or Ala; position X183 is Lys or Glu; position X192 is Leu or Val; position X211 is Met or Thr; position X215 is His or is deleted; position X216 is Ser or is deleted; position X217 is Phe or is deleted; position X231 is Thr or Ser; position X288 is Lys or Glu; position X290 is Glu or Gln, and wherein said sequence is a non naturally-occurring sequence. Further, some such polypeptides have at least one of the properties of CD28 polypeptides described herein, including an ability to enhance an immune response, induce T cell activation or proliferation, exhibit a CD28/CTLA-4 binding affinity ratio about equal to or greater than that of hB7-1, and/or alter cytokine production. For some such polypeptides, the induced T cell response is about equal to or greater than that of hB7-1.

In a preferred embodiment, some such polypeptides comprise two, three, four, five, six, eight, ten, or more of the following amino acids: Thr at position X9; Ile at position X35; Asn at position X55; Leu at position X110; Asp at position X124; Thr at position X135; Lys at position X183; Leu at position X192; Met at position X211; His at position X215; Ser at position X216; Phe at position X217; Thr at position X231; Lys at position X288; and Glu at position X290. In yet another preferred embodiment, some such polypeptides comprise a sequence of any one of SEQ ID NOS:48, 182, 183, 212, 214, 216, 218, 221, and 293.

In another aspect, the invention provides isolated or recombinant polypeptides comprising an amino acid sequence corresponding to an extracellular domain,

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wherein said amino acid sequence has at least about 92% or about 95% amino acid sequence identity to the amino acid sequence corresponding to the extracellular domain of SEQ ID NO:66, and wherein said polypeptide has a CD28/CTLA-4 binding affinity ratio greater than the CD28/CTLA-4 binding affinity ratio of human B7-1 or an ability to induce a T cell proliferation or activation response that is greater than or about equal to that induced by hB7-1. Some such isolated or recombinant polypeptides further comprise at least one further amino acid sequence corresponding to a signal peptide. Some such isolated or recombinant polypeptides further comprise at least one further amino acid sequence corresponding to a transmembrane domain or a cytoplasmic domain.

The invention also provides isolated or recombinant polypeptide variants, each polypeptide comprising an amino acid sequence that differs from the ECD amino acid sequence of B7-1 polypeptide of an Artiodactyla mammal, such as a bovine B7-1, wherein the difference between the amino acid sequence of the variant and the ECD amino acid sequence of the Artiodactyla mammal B7-1 (e.g., bovine B7-1) comprises a different amino acid at one or more of the following amino acid residue positions: position 110, 124, 135, 192, 197, 199, 211, 213, 217, 218, 221, 225, 227, 231, 233, 235, 236, 237, 239, 240, 242, 243, and 244, wherein each position corresponds to the position in the amino acid sequence of the bovine B7-1 of SEQ ID NO:280. An Artiodactyla mammal includes cloven-hoofed mammals, such as bovine, sheep, goats, camels, pigs, deer, giraffes, and antelope (www.ucmp.berkeley.edu/mammal/eutheria/ungulate.html). Some such polypeptide variants comprise variants of full-length bovine B7-1 polypeptide.

For some such polypeptide variants, the difference between the amino acid sequence of the variant and the ECD amino acid sequence of the Artiodactyla B7-1 (e.g., bovine B7-1) comprises at least one of: (a) a different amino acid at position 110 which is Pro; (b) a different amino acid at position 124 which is Val; (c) a different amino acid at position 135 which is Ala; (d) a different amino acid at position 192 which is Val; (e) a different amino acid at position 197 which is Gly; (f) a different amino acid at position 199 which is Glu; (g) a different amino acid at position 211 which is Val; (h) a different amino acid at position 213 which is Asn; (i) a different amino acid at position 217 which is Ile; (j) a different amino acid at position 218 which is Val; (k) a different amino acid at position 221 which is Ile; (l) a different amino acid at position 225 which is Glu; (m) a different amino acid at position 227 which is Ser; (n) a different amino acid at position 231 which is Ile; (o) a different amino acid at position 233 which is Pro; (p) a different amino acid at

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position 235 which is Ser; (q) a different amino acid at position 236 which is Lys; (r) a different amino acid at position 237 which is Pro; (s) a different amino acid at position 239 which is Gln; (t) a different amino acid at position 240 which is Glu; (u) a different amino acid at position 242 which is Pro; (v) a different amino acid at position 243 which is Ile; and (w) a different amino acid at position 244 which is Asp.

For some such polypeptide variants, the difference between the amino acid sequence of the variant and the ECD amino acid sequence of the Artiodactyla B7-1 (e.g., bovine B7-1) further comprises at least one of: (1) a different amino acid at position 246 which is Leu; (2) a different amino acid at position 247 which is Pro; (3) a different amino acid at position 248 which is Phe; (4) a different amino acid at position 250 which is Val; and (5) a different amino acid at position 253 which is Pro, wherein each position corresponds to the position in the amino acid sequence of bovine B7-1 of SEQ ID NO:280. Some such polypeptide variants further comprise a signal peptide (e.g., of a WT mammalian B7-1 or NCSM polypeptide described herein). Some such polypeptide variants also comprise a transmembrane domain and/or cytoplasmic domain, including, e.g., a TMD and/or CD of a WT mammalian B7-1 or NCSM polypeptide described herein.

In some such variants of bovine B7-1, the amino acid in SEQ ID NO:278 at at least one of positions 254, 255, and 256 is deleted. Some such bovine B7-1 variants further comprise at least one of: (6) a different amino acid at position 257 which is Val; (7) a different amino acid at position 258 which is Ser; (8) a different amino acid at position 260 which is Ala; (9) a different amino acid at position 261 which is Leu; (10) a different amino acid at position 263 which is Leu; (11) a different amino acid at position 264 which is Thr; (12) a different amino acid at position 267 which is Val; (13) a different amino acid at position 269 which is Tyr; (14) a different amino acid at position 272 which is Ala; (15) a different amino acid at position 275 which is His; (16) a different amino acid at position 276 which is Val; (17) a different amino acid at position 275 which is His; wherein each position corresponds to the position in the amino acid sequence of SEQ ID NO:280.

Some such bovine B7-1 variants further comprise at least one of: (18) a different amino acid at position 276 which is Val; (19) a different amino acid at position 278 which is Arg; (20) a different amino acid at position 279 which is Trp; (21) a different amino acid at position 280 which is Lys; (22) a different amino acid at position 281 which is Arg; (23) a different amino acid at position 282 which is Thr; (24) a different amino acid at position 284 which is Arg; (25) a different amino acid at position 287 which is Glu; (26) a

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different amino acid at position 288 which is Thr; (27) a different amino acid at position 289 which is Val; (28) a different amino acid at position 290 which is Gly; (29) a different amino acid at position 291 which is Thr; (30) a different amino acid at position 292 which is Glu; (31) a different amino acid at position 293 which is Arg; (32) a different amino acid at position 294 which is Leu; wherein each position corresponds to the position in the amino acid sequence of SEQ ID NO:280.

Some of the above-described polypeptide variants of Artiodactyla mammalian B7-1 (e.g., bovine B7-1) further comprise the following amino acid sequence at the C terminus: IYLGSAQSSG.

Some of the above-described polypeptide variants of Artiodactyla mammalian B7-1 (e.g., bovine B7-1) exhibit a CD28/CTLA-4 binding affinity ratio that is equal to or greater than that of hB7-1 and/or have an ability to induce a T cell proliferation or activation response about equal to or greater than that induced by hB7-1.

The invention also includes nucleic acid sequences (e.g., DNA and RNA) that encode all aforementioned CD28BP polypeptides and Artiodactyla polypeptide variants, and fragments thereof having one or more of the properties described above, and complementary nucleic acid sequences thereof; and expression vectors comprising all such nucleic acid sequences, and cells transformed with such expression vectors.

CTLA-4BP Polypeptides

In one aspect, the invention provides isolated or recombinant CTLA-4BP polypeptides each comprising an amino acid sequence having at least about 85%, 88%, 89%, 90%, 91%, 92&, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more percent identity to at least one of SEQ ID NOS:69-92, 222-252, 286-289, or a subsequence thereof comprising the extracellular domain, wherein said sequence (a) is a non naturally-occurring sequence, and (b) comprises at least one of: Gly at position 2; Thr at position 4; Arg at position 5; Gly at position 8; Pro at position 12; Met at position 25; Cys at position 27; Pro at position 29; Leu at position 31; Arg at position 40; Leu at position 52; His at position 65; Ser at position 78; Asp at position 80; Tyr at position 87; Lys at position 120; Asp at position 122; Lys at position 129; Met at position 135; Phe at position 150; Ile at position 160; Ala at position 164; His at position 172; Phe at position 174; Leu at position 176; Asn at position 178; Asn at position 186; Glu at position 194; Gly at position 196; Thr at position 199; Ala at position 210; His at position 212; Arg at position 219; Pro at position 234; Asn at position 241; Leu at position 244; Thr at position 250; Ala at position 254; Tyr

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at position 265; Arg at position 266; Glu at position 273; Lys at position 275; Ser at position 276; an amino acid deletion at position 276; or Thr at position 279, wherein the position number corresponds to that of hB7-1 amino acid sequence (SEQ ID NO:278), wherein said polypeptide has a CTLA-4/CD28 binding affinity ratio about equal to or greater than a

5   CTLA-4/CD28 binding affinity ratio of hB7-1.

Such CTLA-4BP polypeptides described above have an altered binding affinity for CTLA-4 and/or CD28 as compared to the binding affinity of a WT co-stimulatory molecule for CD28 or CTLA-4. Some such polypeptides have a CTLA-4/CD28 binding affinity ratio about equal to or greater than that of hB7-1. In one aspect, some such

10   polypeptides have a decreased binding affinity for CD28 or CTLA-4 as compared to a binding affinity of a hB7-1 to CD28 or CTLA-4, respectively. Some such polypeptides may inhibit at least one or both of T-cell proliferation or activation in association with co-stimulation of TCR/CD3 (by, e.g., an antigen or anti-CD3 antibody). The induced T-cell proliferative response may be less than that of hB7-1 for some such polypeptides. In

15   another aspect, some such polypeptides described above modulate T-cell activation, but do not induce proliferation of purified T-cells activated by soluble anti-CD3 mAbs.

Some such CTLA-4BP polypeptides each comprise an amino acid sequence having at least about 98% identity to at least one of SEQ ID NOS:69-92, 222-252, and 286-289, said sequence comprising at least one of : Gly at position 2; Gly at position 8; Cys at

20   position 27; His at position 65; Asp at position 80; Asp at position 122; Met at position 135; Phe at position 150; Ala at position 164; Phe at position 174; Asn at position 186; Glu at position 194; Arg at position 219; Thr at position 250; Arg at position 266; Lys at position 275; and Ser at position 276, wherein amino acid position numbers correspond to that of the hB7-1 amino acid sequence (SEQ ID NO:278). In one aspect, such polypeptides may

25   comprise the ECD or full-length amino acid sequence of any one of SEQ ID NOS:69-92, 222-252, and 286-289.

In a preferred embodiment, some such above-described CTLA-4BP polypeptides comprise an amino acid sequence having at least about 98% identity to the extracellular domain of at least one of SEQ ID NOS:69-92, 222-252, and 286-289, said

30   sequence comprising at least one of : His at position 65; Asp at position 80; Asp at position 122; Met at position 135; Phe at position 150; Ala at position 164; Phe at position 174; Asn at position 186; Glu at position 194; and Arg at position 219, wherein the amino acid position numbers correspond to that of hB7-1 amino acid sequence (SEQ ID NO:278).

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Further, some such polypeptides comprise a sequence having at least about 98% identity to the ECD of at least one of SEQ ID NOS:69-92, 222-252, 286-289, said sequence comprising at least two, three, four, five, six or more of: His at position 65; Asp at position 80; Asp at position 122; Met at position 135; Phe at position 150; Ala at position

5   164; Phe at position 174; Asn at position 186; Glu at position 194; and Arg at position 219, wherein the amino acid position numbers correspond to that of h B7-1 amino acid sequence (SEQ ID NO:278). Some such CTLA-4BP polypeptides comprise an ECD amino acid sequence of any one of SEQ ID NOS:69-92 and 222-247. In a preferred embodiment, such CTLA-4BP polypeptides comprise an ECD sequence of any one of SEQ ID NOS:81, 85,

10   86, 88, 90, and 91.

In another aspect, some such above-described CTLA-4BP polypeptides comprises an ECD domain sequence encoded by a coding polynucleotide sequence, the coding polynucleotide sequence selected from the group: (a) an ECD coding sequence of a polynucleotide sequence selected from any of SEQ ID NOS:22-45 and 143-173; (b) a

15   polynucleotide sequence that encodes the ECD amino acid sequence of a polypeptide selected from any of SEQ ID NOS:69-92, 222-252, and 286-289; and (c) a polynucleotide sequence which, but for the codon degeneracy, hybridizes under stringent conditions over substantially the entire length of a polynucleotide sequence (a) or (b).

Some such above-described isolated or recombinant polypeptides, including,

20   but not limited to, those having the binding and/or proliferation properties discussed above, further comprise a signal peptide amino acid sequence encoded by a signal peptide coding nucleotide sequence, the signal peptide coding nucleotide sequence selected from the group of: (a) a nucleotide sequence comprising a nucleotide fragment of a polynucleotide sequence selected from any of the group of SEQ ID NOS:22-45 and 143-173, wherein said

25   nucleotide fragment encodes a signal peptide; (b) a nucleotide sequence that encodes the signal peptide of a polypeptide selected from any of the group of SEQ ID NOS:69-92, 222-252, and 286-289; and (c) a nucleotide sequence which, but for codon degeneracy, hybridizes under at least stringent conditions over substantially the entire length of a nucleotide sequence (a) or (b).

30   Some of the above-described isolated or recombinant polypeptides further comprising a transmembrane domain (TMD) amino acid sequence encoded by a TMD nucleotide sequence selected from the group of: (a) a nucleotide sequence of a polynucleotide sequence selected from any of the group of SEQ ID NOS:22-45 and 143-

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173, wherein said nucleotide sequence encodes a TMD polypeptide; (b) a nucleotide sequence that encodes the TMD of a polypeptide selected from any of the group of SEQ ID NOS:69-92, 222-252, and 286-289; and (c) a nucleotide sequence which, but for codon degeneracy, hybridizes under at least stringent conditions over substantially the entire

5   length of a nucleotide sequence (a) or (b). Some such polypeptides further comprise a cytoplasmic domain (CD) amino acid sequence encoded by a CD nucleotide sequence selected from the group of: (a) a nucleotide sequence of a polynucleotide sequence selected from any of the group of SEQ ID NOS:22-45 and 143-173, wherein said nucleotide sequence encodes a CD polypeptide; (b) a nucleotide sequence that encodes the CD of a

10   polypeptide selected from any of the group of SEQ ID NOS:69-92, 222-252, and 286-289, and 290-293; and (c) a nucleotide sequence which hybridizes under at least stringent conditions over substantially the entire length of a nucleotide sequence (a) or (b).

The invention includes isolated or recombinant polypeptides each comprising an amino acid sequence that differs from a primate B7-1 sequence in at least one

15   of the mutation selected from the following at an amino acid residue position of indicated, wherein the position corresponds to the amino acid position within the amino acid sequence of hB7-1 as shown in SEQ ID NO:278: 1) 40 Arg; 52 Leu; 65 His; 122 Asp; 129 Lys; 135 Met; 164 Ala; 174 Phe; 196 Gly; 199 Thr; 210 Ala; 219 Arg; 234 Pro; 241 Asn; or 2) 12 Pro; 25 Met; 27 Cys; 29 Pro; 40 Arg; 52 Leu; 65 His; 122 Asp; 129 Lys; 135 Met; 164 Ala;

20   174 Phe; 196 Gly; 199 Thr; 210 Ala; 219 Arg; 234 Pro; 241 Asn; 254 Ala; 275 Lys; 276 Ser; or 279 Thr. The invention also provides isolated or recombinant polypeptides each comprising an amino acid sequence that differs from a primate B7-1 sequence in at least one mutation selected from: Ser 12 Pro; Leu 25 Met; Gly 27 Cys; Ser 29 Pro; Lys 40 Arg; His 52 Leu; Tyr 65 His; Glu 122 Asp; Glu 129 Lys; Thr 135 Met; Thr 164 Ala; Ser 174 Phe;

25   Glu 196 Gly; Ala 199 Thr; Thr 210 Ala; Lys 219 Arg; Thr 234 Pro; Asp 241 Asn; Val 254 Ala; Arg 275 Lys; Arg 276 Ser; or Arg 279 Thr. The mutation being indicated is relative, e.g., to human B7-1 with the amino acid sequence shown in SEQ ID NO:278, the sequence does not occur in nature, and, in some sequences, the polypeptide has a CTLA-4/CD28 binding affinity ratio about equal to, equal to or greater than the CTLA-4/CD28 binding

30   affinity ratio of human B7-1. Each amino acid position corresponds to the position of the amino acid sequence of SEQ ID NO:278. In some embodiments, the sequence of some such polypeptides differs from primate B7-1 sequence in at least two of said mutations. In

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some aspects, the primate B7-1 is hB7-1 (SEQ ID NO:278), and in some aspects, the sequence differs from the hB7-1 sequence in at least two mutations.

In another aspect, the invention provides isolated or recombinant CTLA-4BP polypeptides comprising an amino acid sequence having at least about 75%, 80%, 85%, 90%, 95%, or more percent identity to at least one of SEQ ID NOS:263-272, or a subsequence thereof comprising the ECD, wherein the sequence is not a naturally-occurring, and the polypeptide has a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of hB7-1 or an ability to induce a T cell proliferation or activation response about equal to or less than that of hB7-1.

In yet another aspect, the invention provides isolated or recombinant polypeptides that each comprise a non naturally-occurring amino acid sequence encoded by a nucleic acid comprising a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:22-45, 143-173, 253-262, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:69-92, 222-247, 263-272, 286-289, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which hybridizes under at least stringent or highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b); (d) a polynucleotide sequence comprising all or a fragment of (a), (b), or (c), wherein the fragment encodes a polypeptide having a CTLA-4/CD28 binding affinity ratio about equal to or greater than that of hB7-1; (e) a polynucleotide sequence encoding a polypeptide, the polypeptide comprising an amino acid sequence which is substantially identical over at least about 150, 180, 200, 225, 250 or more contiguous amino acid residues of any one of SEQ ID NOS:69-92, 222-247, 263-272, 286-289, and (f) a polynucleotide sequence encoding a polypeptide that has a CTLA-4/CD28 binding affinity ratio about equal to or greater than that of hB7-1, which polynucleotide sequence has at least about 70%, 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 99%, or more identity to at least one polynucleotide sequence of (a), (b), (c), or (d). Some such polypeptides comprise an amino acid sequence of any one of SEQ ID NOS:69-92, 222-247, 263-272, and 286-289.

Such above-described polypeptides have a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1. Some such polypeptides inhibit T-cell proliferation. The induced T-cell response may be less than that of human B7-1.

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In yet another aspect, the invention includes isolated or recombinant polypeptides that each comprise a sequence according to the formula:

MGHTRRQGTSP-X12-KCPYLKFFQLLV-X25-ACL-X29-HLCSGVIHVT-X40-EVKEVATLSCGLNVSVEELAQTRIHWQKEKKMVLTM MSGDMNIWPEYKNRTIFDITNNLSIVILALRPSDEGTYECVVLKY-X122-KDAFKR-X129-HLAEVMLSVKAD FPTPSITDFEIPPSNIRRIICS-X164-SGGFPEPHLFWLENGEELNAINTTVSQDPET-X196-LYTVSSKLDFNM TANHSFMCLI-X219-YGHLRVNQTFNWNTPKQEHFP-X241-NLLPSWA ITLISANGIFVICCLTYRFAPRCRERKSNETLRRESVCPV (SEQ ID NO:287), or a subsequence thereof comprising the extracellular domain, wherein position X12 is Ser or Pro; position X25 is Leu or Met; position X29 is Ser or Pro; position X40 is Lys or Arg; position X122 is Glu or Asp; position X129 is Glu or Lys; position X164 is Thr or Ala; position X196 is Glu or Gly; position X219 is Lys or Arg; and position X241 is Asp or Asn. In a preferred embodiment, some such polypeptides comprise the ECD of SEQ ID NO:288 or SEQ ID NO:289. In another preferred embodiment, some such polypeptides comprise the amino acid sequence SEQ ID NO:288 or SEQ ID NO:289. In one aspect, such polypeptides exhibit at least one of the CTLA-4BP properties described above, including a CTLA-4/CD28 binding affinity ratio about equal to or greater than the CTLA-4/CD28 binding affinity ratio of human B7-1. Some such polypeptides inhibit T-cell proliferation; for some polypeptides, the induced T-cell response is less than that induced by hB7-1 in the presence of e.g., anti-CD3 Abs or antigen.

The invention also provides isolated or recombinant polypeptides that each comprise a subsequence of an amino acid sequence set forth in any of SEQ ID NOS:69-92, 222-247, 263-272, and 286-289, wherein the subsequence is the ECD of the amino acid sequence.

In addition, the invention provides novel isolated or recombinant polypeptides corresponding to baboon and orangutan B7-1. Such polypeptides comprise the sequence SEQ ID NO:93 or SEQ ID NO:94, or a subsequence thereof, wherein the subsequence comprises at least one of: the signal sequence, extracellular domain, transmembrane domain, and the cytoplasmic domain of the polypeptide.

Also included are nucleic acid sequences (e.g., DNA and RNA) that encode all aforementioned CTLA-4BP polypeptides and fragments thereof having one or more of

the properties described above, and expression vectors comprising such nucleic acid sequences, and cells transformed with such expression vectors.

B7-1 and B7-2 Polypeptide Variants

The invention also provides polypeptide variants of a WT or mutant B7-1 polypeptide, including, e.g., polypeptide variants of the hB7-1 polypeptide shown in SEQ ID NO:278 or other parental primate B7-1 polypeptide described herein (e.g., SEQ ID NOS:93-94, 279-282). Each such variant comprises an amino acid sequence that differs from the amino acid sequence of the WT or mutant (reference) B7-1 by at least one amino acid residue. In one aspect, the polypeptide variant comprises an amino acid sequence that differs from that of a full-length WT or mutant (reference) B7-1 polypeptide by substitution in said full-length B7-1 polypeptide of at least one amino acid residue at a position that corresponds to position 65 of the hB7-1 polypeptide sequence shown in SEQ ID NO:278. The substituted amino acid residue at this position may comprise any amino acid residue that differs from that in the reference sequence. In another aspect, the substituted amino acid at this position is any amino acid residue other than alanine or an amino acid residue existing at that position in a known primate B7-1 polypeptide sequence. In another aspect, the substituted amino acid at this position is an amino acid residue selected from the group consisting of histidine (His), arginine (Arg), lysine (Lys), proline (Pro), phenylalanine (Phe), and tryptophan (Trp). Polypeptide variants of the invention include amino acid sequences that differ from the amino acid sequence of a WT or mutant primate B7-1 polypeptide by at least one amino acid substitution, wherein the at least one amino acid substitution comprises a substitution in the amino acid sequence of said primate B7-1 for the amino acid position that corresponds to position 65 of SEQ ID NO:278 with any amino acid residue selected from the group of His, Arg, Lys, Pro, Phe, and/or Trp. Usually, the substituted amino acid is His, and the amino acid for which His is substituted is Tyr. Polypeptide variants also include amino acid sequences that differ from the amino acid sequence of SEQ ID NO:278 by the substitution of the amino acid residue in SEQ ID NO:278 (e.g., Tyr) at position 65 with any amino acid residue selected from the group of His, Arg, Lys, Pro, Phe, and Trp. Preferably, the polypeptide variant comprises an amino acid sequence that differs from that of SEQ ID NO:278 by at least one amino acid substitution comprising the substitution of His for Tyr at position 65 in SEQ ID NO:278 (Tyr65His substitution).

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The invention also provides polypeptide variants of a polypeptide fragment or segment of a full-length WT or mutant (reference) B7-1 polypeptide, including, but not limited to, a primate B7-1 polypeptide. In this aspect, the polypeptide variant comprises an amino acid sequence that differs from a first amino acid sequence, which comprises an

5   amino acid fragment or segment of a full-length amino acid sequence of a primate B7-1, by at least one amino acid residue. The amino acid fragment typically comprises a signal peptide and/or ECD polypeptide or a mature domain of the primate B7-1 amino acid sequence. Some such polypeptide variants comprise an amino acid sequence that differs from said first amino acid sequence comprising a signal peptide and/or ECD or a mature

10   domain of a primate B7-1 sequence by substitution in said first amino acid sequence of at least one amino acid residue at a position that corresponds to position 65 of hB7-1 (SEQ ID NO:278). The substituted amino acid at this position may comprise any amino acid residue that differs from that in the first amino acid sequence. Usually, the substituted amino acid comprises an amino acid other than alanine or an amino acid residue existing at that position

15   in a primate B7-1 polypeptide sequence. Preferably, the substituted amino acid at this position comprises an amino acid residue selected from the group consisting of His, Arg, Lys, Pro, Phe, and/or Trp.

Also included are polypeptide variants comprising amino acid sequences that differ from a first amino acid sequence, wherein the first amino acid sequence comprises a

20   signal peptide sequence and/or ECD polypeptide sequence of a primate B7-1 polypeptide sequence (including, e.g., hB7-1), and wherein the variant that differs from said first amino acid sequence by the substitution of at least one amino acid residue in the first amino acid sequence at a position corresponding to position 65 of the amino acid sequence of SEQ ID NO:278. In one such aspect, the substituted amino acid residue is selected from the group

25   of His, Arg, Lys, Pro, Phe, and/or Trp. Usually, the polypeptide variant comprises an amino acid sequence that differs from an amino acid sequence comprising amino acids 1-243 of SEQ ID NO:278 by the substitution of His for Tyr at position 65 of SEQ ID NO:278 (Tyr65His substitution).

B7-1 polypeptide variants of a full-length primate B7-1 polypeptide or

30   polypeptide fragments thereof (e.g., corresponding to a signal peptide and/or ECD or mature domain of a primate B7-1 as described above) have an altered binding activity compared with the binding activity of the full-length primate B7-1 polypeptide sequence (or polypeptide fragment thereof as described above) or an altered binding affinity ratio

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compared with the binding affinity ratio of the full-length primate B7-1 polypeptide sequence (or polypeptide fragment thereof as described above). Some such polypeptide variants of a primate B7-1 polypeptide (or fragment thereof described above) do not bind a CD28-Ig (Figs. 23A-23B) as does hB7-1, but they do bind CTLA-4-Ig in a manner that is

5 substantially identical or equivalent to that of hB7-1, or in a manner that produces a binding profile substantially identical or equivalent to that of hB7-1. Some such polypeptide variants have a CTLA-4/CD28 binding affinity ratio that is about equal to or greater than the CTLA-4/CD28 binding affinity ratio of a primate B7-1 (e.g., hB7-1), under the conditions described in Figs. 23-24.. In addition, some such variants induce a decreased

10 level of T cell proliferation compared to the level of T cell proliferation induced by hB7-1 or CD28BP-15 (Figs. 23A-23B).

The invention includes a polypeptide variant of a WT or mutant B7-1 having an altered binding activity or altered binding affinity ratio compared with the binding activity or binding affinity ratio of a first B7-1 polypeptide or polypeptide fragment thereof

15 (e.g., a polypeptide fragment corresponding to a signal peptide and/or ECD or mature domain of the first B7-1 polypeptide as described above), wherein the polypeptide variant has an amino acid sequence that differs from the amino acid sequence of the first B7-1 polypeptide (or polypeptide fragment thereof) by at least about 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% homologous or identical with the amino

20 acid sequence of the full-length hB7-1 polypeptide sequence (i.e., the second polypeptide) shown in SEQ ID NO:278 or with the amino acid sequence of a polypeptide fragment of SEQ ID NO:278, such as, e.g., a fragment corresponding to a signal peptide and/or ECD or mature domain (e.g., a fragment comprising amino acid residues 1-243, 35-243, or 35-288 of SEQ ID NO:278, respectively). The difference between the amino acid sequence of the

25 variant and the amino acid sequence of the first B7-1 polypeptide (or fragment thereof) comprises a different amino acid at a position corresponding to position 65 of the amino acid sequence of SEQ ID NO:278. In one aspect, the different amino acid is selected from the group of His, Arg, Lys, Pro, Phe, and/or Trp, and, preferably, comprises His. The first B7-1 polypeptide may comprise a WT B7-1 or a mutant, derivative, or conservatively

30 substituted variant of the WT B7-1, and some such variants induce a decreased level of T cell proliferation or lack T cell proliferation compared to the level of T cell proliferation induced by hB7-1.

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The invention provides a polypeptide variant of a WT or mutant B7-2 polypeptide, including, but not limited to, e.g., a polypeptide variant of human B7-2 (hB7-2) or other primate B7-2 polypeptide, wherein the variant comprises an amino acid sequence that differs from the amino acid sequence of a WT or mutant hB7-2 by at least one

5 amino acid residue. The full-length polypeptide and nucleic acid sequences of a WT (reference) hB7-2 are provided in GenBank at GenBank Accession Nos. AAA58389 and L25259, respectively (see Freeman, G.J. et al., (1993) Science 262:909-911). The full-length polypeptide and nucleic acid sequences of a second WT (reference) hB7-2 are provided at GenBank Accession Nos. AAA86473 and U17717, respectively (see Azuma,

10 M. et al., (1993) Nature 366:76-79). In one aspect, the polypeptide variant comprises an amino acid sequence that differs from that of a full-length WT or mutant B7-2 (or fragment thereof comprising a signal peptide and/or ECD or mature domain of the full-length B7-2) polypeptide by substitution in said full-length B7-2 polypeptide (or fragment thereof) of at least one amino acid residue at an amino acid position corresponding to: 1) position 65 of

15 the hB7-1 polypeptide sequence shown in SEQ ID NO:278; 2) position 56 of the B7-2 polypeptide sequence shown at GenBank Acc. No. AAA58389; or 3) position 50 of the B7-2 polypeptide sequence shown at GenBank Acc. No. AAA86473. Usually, the substituted amino acid at this position is an amino acid residue selected from the group consisting of His, Arg, Lys, Pro, and/or Trp. In some aspect, the substituted amino acid replaces

20 phenylalanine in B7-2. The B7-2 polypeptide variant may comprise a modified amino acid sequence comprising the B7-2 amino acid sequence shown at GenBank Acc. No. AAA58389 (or an amino acid fragment thereof comprising a signal peptide and/or ECD or mature domain of said B7-2 sequence) that has been modified by a Phe56His substitution. The B7-2 polypeptide variant may comprise a modified amino acid sequence comprising

25 the B7-2 amino acid sequence shown at GenBank Acc. No. AAA86473 (or an amino acid fragment thereof comprising a signal peptide and/or ECD or mature domain of said B7-2 sequence) that has been modified by a Phe50His substitution.

Some such B7-2 polypeptide variants of a full-length primate B7-2 polypeptide (or polypeptide fragment thereof, such as, e.g., that which corresponds to a

30 signal peptide and/or ECD or mature domain of a primate B7-2) have an altered binding activity compared with the binding activity of the full-length (reference) primate B7-2 polypeptide sequence (or polypeptide fragment thereof) or an altered binding affinity ratio compared with the binding affinity ratio of the full-length primate B7-2 polypeptide

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sequence (or polypeptide fragment thereof). Some such polypeptide variants of a primate B7-2 polypeptide (or fragment thereof) bind a CTLA-4 receptor with an equal or greater binding affinity than does the primate B7-2 polypeptide (or fragment thereof) and/or bind a CD28 receptor with an equal or lesser binding affinity than does the primate B7-2 (or

5   fragment thereof). Some B7-2 polypeptide variants have a CTLA-4/CD28 binding affinity ratio that is about equal to or greater than the CTLA-4/CD28 binding affinity ratio of a primate B7-2 polypeptide sequence or fragment thereof. Some such variants induce a decreased level of T cell proliferation compared to the level of T cell proliferation induced by hB7-2 and/or CD28BP-15.

10   Also included is a polypeptide variant of a WT or mutant B7-2 having an altered binding activity or altered binding affinity ratio compared with the binding activity or binding affinity ratio of a first B7-2 polypeptide or polypeptide fragment thereof (e.g., a polypeptide fragment corresponding to a signal peptide and/or ECD or mature domain of the first B7-2 polypeptide), under the conditions described in Example IX (see, e.g., Figs.

15   23-24), wherein the B7-2 polypeptide variant has an amino acid sequence that differs from the amino acid sequence of the first B7-2 polypeptide (or polypeptide fragment thereof) and is at least about 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% homologous or identical with the amino acid sequence of the hB7-2 polypeptide sequence (i.e., the second polypeptide) shown at GenBank Acc. No. AAA58389 or

20   AAA86473, or with the amino acid sequence of a polypeptide fragment of either of such B7-2 sequence shown in GenBank. The difference between the amino acid sequence of the B7-2 polypeptide variant and the amino acid sequence of the first B7-2 polypeptide (or fragment thereof) comprises a different amino acid at a position corresponding to: 1) position 65 of the amino acid sequence of hB7-1 shown in SEQ ID NO:278; 2) position 56

25   of the hB7-2 polypeptide sequence at GenBank Acc. No. AAA58389; or 3) position 50 of the hB7-2 polypeptide sequence at GenBank Acc. No. AAA86473. The different amino acid may be selected from the group of His, Arg, Lys, Pro, and/or Trp, and preferably comprises His. The first B7-2 polypeptide may comprise a WT B7-2 or a mutant, derivative, or conservatively substituted variant of the WT B7-2. Some variants induce a

30   decreased level of T cell proliferation compared to that induced by hB7-2 and/or CD28BP-15.

            Also included are nucleic acid sequences (e.g., DNA and RNA) that encode all aforementioned B7-1 and B7-2 polypeptide variants and fragments thereof having one or

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more of the properties described above, and expression vectors comprising such nucleic acid sequences, and cells transformed with such expression vectors. Degenerate nucleotide sequences of such nucleotide variants are also a part of the invention. Also included are nucleotide variants (e.g., DNA or RNA variants) of a nucleic acid sequence encoding a WT or mutant B7-1 polypeptide, including, but not limited to, e.g., a nucleic acid variant of a nucleotide sequence encoding a (reference) hB7-1 polypeptide (SEQ ID NO:273) or other parental primate B7-1 described herein (SEQ ID NOS:46-47, 274-277) or a fragment thereof comprising, e.g., a signal peptide and/or ECD polypeptide or a mature domain of the (reference) human or primate B7-1. Each such variant comprises a nucleic acid sequence that differs from the nucleic acid sequence of a WT or mutant (reference) B7-1 nucleic acid sequence by at least one nucleic acid residue. The nucleic acid variant may comprise a nucleic acid sequence that differs from a first nucleic acid sequence encoding a full-length WT or mutant B7-1 polypeptide (or polypeptide fragment thereof) by substitution in said first nucleic acid sequence of at least one different nucleic acid residue at a position that corresponds to one of the three nucleic acid residues in the codon that encodes the amino acid corresponding to the amino acid at position 65 of the hB7-1 polypeptide of SEQ ID NO:278. The modified codon usually encodes an amino acid selected from the group of His, Arg, Lys, Pro, Phe, and/or Trp, and, typically, His. All codons encoding such amino acids are well known. In another aspect, the nucleic acid variant comprises a nucleic acid sequence that differs from a first nucleic acid sequence that encodes a full-length WT or mutant B7-1 polypeptide (or polypeptide fragment thereof) by at least one nucleic acid substitution in said first nucleic acid sequence, wherein said at least one nucleic acid substitution comprises a substitution of a cytosine (C) for a thymine (T) at position 193 in SEQ ID NO:273.

In one aspect, the nucleic acid comprises: (a) a polynucleotide sequence comprising the polynucleotide sequence of SEQ ID NO:273 in which the 3 nucleic acid residues TAC at positions 193-195 are replaced by the three nucleic acid residues CAC, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide of SEQ ID NO:278, in which the codon comprising 3 nucleotide residues encoding Tyr are replaced by a codon comprising three nucleotide residues encoding His, or a complementary polynucleotide sequence thereof; and (c) a polynucleotide sequence comprising all or a nucleotide fragment of (a) or (b), wherein the nucleotide fragment encodes a polypeptide having a CTLA-4/CD28 binding affinity ratio about equal to or

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greater than the CTLA-4/CD28 binding affinity ratio of human B7-1 or a polypeptide having an ability to induce a T cell proliferation response that is less than that induced by human B7-1.

In addition, the invention includes nucleic acid variants (e.g., DNA or RNA variants) of a nucleic acid sequence encoding a WT or mutant B7-2 polypeptide, including, but not limited to, a nucleic acid variant of the (reference) nucleotide sequence shown at GenBank Acc. No. AAA86473 and U17717, or a nucleic acid variant of a nucleotide sequence encoding a (reference) hB7-2 polypeptide (GenBank Acc. No. AAA58389 or AAA86473) or other primate B7-2 sequence, or a polypeptide fragment thereof comprising, e.g., a signal peptide and/or ECD polypeptide or a mature domain of the (reference) human or primate B7-2. Each such variant comprises a nucleic acid sequence that differs from the nucleic acid sequence of a WT or mutant (reference) B7-2 nucleic acid sequence by at least one nucleic acid residue. The nucleic acid variant may comprise a nucleic acid sequence that differs from a first nucleic acid sequence encoding a full-length WT or mutant B7-2 polypeptide (or polypeptide fragment thereof) by substitution in said first nucleic acid sequence of at least one different nucleic acid residue at a position that corresponds to one of the three nucleic acid residues in the codon that encodes the amino acid corresponding to the amino acid at: 1) position 65 of the hB7-1 polypeptide of SEQ ID NO:278, 2) position 56 of the hB7-2 polypeptide sequence at GenBank Acc. No. AAA58389; or 3) position 50 of the hB7-2 polypeptide sequence at GenBank Acc. No. AAA86473. The modified codon usually encodes an amino acid selected from the group of His, Arg, Lys, Pro, and/or Trp, and, typically, His. All codons encoding such amino acids are well known. Also provided are nucleic acid sequences (and fragments thereof that encode polypeptides having at least one of the properties set forth above) that, but for codon degeneracy, hybridize under at least stringent or highly stringent conditions to one or more of the B7-1 or B7-2 nucleotide variants (or fragments thereof) described above, and nucleic acid sequences complementary to those described above.

Additional Aspects

The invention also includes a polypeptide comprising an ECD of an NCSM polypeptide (or B7-1 or B7-2 polypeptide variant) of the invention with at least one of a signal peptide, transmembrane domain, and/or cytoplasmic domain. Usually, the transmembrane domain and/or cytoplasmic domain is from a co-stimulatory molecule, such as an NCSM polypeptide of the invention or a B7-1 or B7-2 polypeptide. Any isolated or

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recombinant CD28BP or CTLA-4 polypeptide described above may further comprise at least one of the following components: a signal sequence, transmembrane domain, or cytoplasmic domain. Various combinations of such from the various NCSM polypeptide sequences described herein can be made. In one aspect, a signal sequence, transmembrane

5   domain or cytoplasmic domain signal sequence is selected from the signal sequence, transmembrane domain, or cytoplasmic domain, respectively, set forth in any of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293.

A wide variety of signal peptide sequences can be fused to an ECD of a NCSM polypeptide or a B7-1 or B7-2 polypeptide variant to generate a polypeptide

10   comprising at least a signal peptide sequence and an ECD polypeptide. Signal peptides that can be used include, but are not limited to, the amino acid sequence corresponding to a signal peptide of a WT B7-1 or B7-2 polypeptide, including, but not limited to, a mammalian or primate B7-1 or B7-2 polypeptide, and a signal peptide sequence from tissue plasminogen activator (TPA). The signal peptide sequence facilitates expression of the

15   ECD polypeptide in vitro and in vivo and is typically cleaved from the ECD.

In some embodiments, the signal peptide sequence comprises about 33, about 34, about 35, or about 36 amino acids. For the NCSM polypeptide sequences showing in the sequence listing, the signal peptide is typically about 34 amino acids in length. The ECD of an NCSM polypeptide, such as a CD28BP or CTLA-4BP molecule, or

20   a B7-1 or B7-2 polypeptide variant of the invention typically begins with the first amino acid residue following the last amino acid residue of the signal peptide sequence. The predicted cleavage site of a signal peptide for any of the NCSM polypeptide sequences (and nucleic acid sequences encoding such NCSM polypeptides) described herein can be predicted by methods known to those skilled in the art; see, e.g., Nielsen et al., Protein

25   Eng'g 10:1-6 (1997), www.cbs.dtu.dk/services/SignalP/, and www.cbs.dtu.dk/services/SignalP/bg_prediction.html.

One of ordinary skill in the art would understand that the predicted boundary between the signal peptide and ECD of an NCSM polypeptide (or B7-1 B7-2 polypeptide variant) of the invention can be determined from this alignment by comparing the ECD

30   sequence of WT hB7-1 with the sequence of the NCSM molecule of interest. As shown in Figures 2A-2B, e.g., the signal peptide sequence for WT hB7-1 generally ends with the three amino acid residues -cysteine-serine-glycine (-CSG). As shown by alignment with hB7-1, the signal peptide sequence for many CD28BPs of the invention also ends with the

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residues (-CSG or -SG) (see, e.g., CD28BP-15 (SEQ ID NO:66)). The signal peptide sequence for many CTLA-4BPs ends the residues -cysteine-serine-glycine (-CSG) (Figs. 3A-3B).

The ECD sequence of WT hB7-1 generally begins with the three amino acid residues valine-isoleucine-histidine- (VIH-). The ECD of a CTLA-4BP usually begins with the same VIH- sequence, as shown in the alignment of CTLA-4BP sequences with the hB7-1 sequence (Figs. 3A-3B). The ECD of an NCSM polypeptide (or B7-1 or B7-2 polypeptide variant) may begin with a hydrophobic amino acid residue. For example, in some such aspects, the ECD of a CD28BP begins with hydrophobic residue. In some aspects, the ECD sequence of a CD28BP begins with the three amino acid residues isoleucine-threonine-proline- (ITP-) (see, e.g., CD28BP-15 (SEQ ID NO:66)). In some embodiments, the ECD of a CD28BP begins with a proline residue; in some such embodiments, the ECD begins with the three amino acid residues -PKS. In other embodiments, the ECD of some CD28BPs begins with residues -IS (see, e.g., SEQ ID NOS: 49, 50, 65 and 191). The ECD sequence of many CTLA-4BPs generally begins with amino acid residues valine-isoleucine-histidine- (VIH-).

An ECD NCSM polypeptide (or B7-1 or B7-2 polypeptide variant) may be fused to a signal peptide sequence from a WT B7-1 or WT B7-2 polypeptide, such as a mammalian or primate B7-1 or B7-2 polypeptide. Alternatively, an ECD NCSM polypeptide (or B7-1 or B7-2 polypeptide variant) is fused to an amino acid sequence corresponding to a signal peptide of any NCSM polypeptide of the invention, including, e.g., the signal peptide sequence of SEQ ID NOS:48-94, 174-252, 263-272, and 278-293.

A nucleic acid sequence encoding an ECD NCSM polypeptide (or B7-1 or B7-2 polypeptide variant) may be fused to a nucleic acid sequence encoding a signal peptide. The nucleic acid sequence encoding the signal peptide comprises the nucleic acid sequence encoding a signal peptide of a WT B7-1 or WT B7-2 polypeptide, such as a mammalian or primate B7-1 or B7-2 polypeptide. In another aspect, a nucleotide sequence encoding an ECD of an NCSM polypeptide (or of a B7-1 or B7-2 polypeptide variant) is fused to a nucleic acid sequence encoding a signal peptide sequence, wherein said nucleic acid sequence comprises a signal peptide coding nucleotide sequence of any NCSM nucleic acid or WT B7-1 or B7-2, including, e.g., the signal peptide coding nucleotide sequence of any of SEQ ID NOS:1-47, 95-173, 253-262, and 273-277. In a particular embodiment, the invention provides a nucleic acid sequence comprising encoding a signal peptide and ECD

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comprising amino acid residues 1-244 of SEQ ID NO:66. For one such embodiment, the nucleic acid comprises nucleotide residues 1-732 of SEQ ID NO:19.

The predicted boundary between the ECD and TMD in the hB7-1 sequence is between amino acid residues 242 and 243, based on numbering of the full-length WT hB7-1 sequence (Fig. 2G). The predicted boundary between the ECD and TMD of an NCSM polypeptide (or B7-1 or B7-2 polypeptide variant) can be determined by comparison of the position of the amino acid residues at the predicted ECD/TM boundary of hB7-1 with amino acid residues at the corresponding positions in the NCSM polypeptide of interest. In one embodiment, the predicted ECD/TM boundary of CD28BP-15 (SEQ ID NO:66) is between amino acid residues 244 and 245, the ECD and TMD of CD28BP-15 comprise residues 35-244 and 245-268 of SEQ ID NO:66, respectively. In another embodiment, based on the numbering of amino acid residues in the full-length hB7-1 sequence, the predicted ECD/TM boundary of CTLA-4BP 5x4-12c (SEQ ID NO:86) is between amino acid residues 242 and 243, the ECD and TMD of CTLA-4BP 5x4-12c comprise residues 35-242 and 243-263 of SEQ ID NO:86, respectively.

Alternatively, the predicted boundary between the ECD and TMD of an NCSM polypeptide (or B7-1 or B7-2 polypeptide variant described herein) is determined using a software program known by those of ordinary skill in the art that identifies one or more hydrophobic amino acid residues likely to be present at the beginning of the TMD, after the ECD. The program Vector NTI BioPlot analysis, version 6.0 Protein Scales via ProScales on ExPASy Server (Kyte J., Doolittle R.F., J. Mol. Biol. 157:105-132(1982)) can be used, e.g., to determine hydropathicity regions of a polypeptide sequence and predict, e.g., the transmembrane region of an NCSM polypeptide, B7-1 or B7-2 polypeptide variant of the invention. With this program, the following parameters can be selected: Amino acid scale: Hydropathicity; window size: 9; relative weight of the window edges compared to the window center (in %): 100%; weight variation model (if the relative weight at the edges is < 100%): linear; and the scale need not be normalized from 0 to 1.

In one aspect, the predicted ECD/TM boundary of CD28BP-15 is between amino acid residues 255 and 256, and the ECD and TMD of CD28BP-15 comprise residues 35-255 and 256-272 of a polypeptide selected from the group of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, and preferably SEQ ID NO:66. In another aspect, the TMD comprises at least about amino acid residues 35-244, 35-243, 35-242, 35-255, 35-254, or 35-253 of a polypeptide selected from any of SEQ ID NOS:48-68, 174-221, 283-285, and

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290-293, and preferably SEQ ID NO:66. In one aspect, the CD amino acid sequence comprises a cytoplasmic domain of a polypeptide selected from any of SEQ ID NOS:48-68, 174-221, 283-285, and 290-293, preferably SEQ ID NO:66.

One of skill would recognize, however, that the ECD of a CD28BP, CTLA-4BP, or B7-1 or B7-2 polypeptide variant may contain additional (e.g., one, two, or three) amino acid residues or fewer amino acid residues (e.g., one, two or three residues) at the N-terminus and/or C-terminus and still maintain equivalent or comparable properties as of the molecule as described herein, and that the present invention includes such embodiments. Also included are polypeptides comprising a subsequence of SEQ ID NOS:49 and 50, wherein said subsequence corresponds to an ECD (e.g., co-stimulatory ECD).

Any isolated or recombinant CD28BP or CTLA-4BP polypeptide described above may comprise a soluble extracellular domain of the respective full-length CD28BP or CTLA-4BP polypeptide or a fragment (e.g., truncated ECD) or subsequence thereof. Any such CD28BP or CTLA-4BP polypeptide may comprise a fusion protein comprising a CD28BP-ECD or CTLA-4BP-ECD or fragment thereof and at least one additional amino acid sequence, which may comprise at least one Ig polypeptide. The at least one Ig polypeptide may comprise at least one human IgG polypeptide comprising an Fc hinge, a CH2 domain, and a CH3 domain.

Any isolated or recombinant CD28BP or CTLA-4BP polypeptide described above may also comprise a polypeptide purification subsequence. The polypeptide purification subsequence is selected from, e.g., an epitope tag, a FLAG tag, a polyhistidine sequence, and a GST fusion.

In addition, isolated or recombinant CD28BP or CTLA-4BP polypeptide described above or a B7-1 or B7-2 polypeptide variant may comprise a modified amino acid. The modified amino acid can be, e.g., a glycosylated amino acid, a PEGylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, an amino acid conjugated to a lipid or sugar moiety, polymer, and an amino acid conjugated to an organic derivatizing agent. Methods for modifying one or more amino acids of CD28BP, CTLA-4BP, and/or B7-1 and B7-2 polypeptide variants, including methods of glycosylating and pegylating amino acids are known in the art; additional methods are set forth in PCT Application No. PCT/DK01/00094 (Publ. No. WO 01/58935), which published on August 16, 2001. The invention also provides a composition comprising at least one polypeptide of any CD28BP and/or CTLA-4BP polypeptide described above and

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an excipient or carrier. In one aspect, the composition comprises an isolated or recombinant NCSM polypeptide comprising the amino acid sequence SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, or a fragment thereof and a carrier or excipient. The CD28BP fragment has a CD28/CTLA-4 binding affinity ratio about equal to or greater than the

5   CD28/CTLA-4 binding affinity ratio of human B7-1 and/or an ability to induce a T cell response about equal to or greater than that induce by hB7-1. The CTLA-4BP fragment has a CTLA-4/CD28 binding affinity ratio about equal to or greater than that of h7-1. The composition may be a pharmaceutical composition including a pharmaceutically acceptable excipient or carrier. Exemplary and preferred compositions and pharmaceutically

10   acceptable excipients and carriers are described below.

<center>Consensus Sequences and Subsequences</center>

The present invention also includes at least one NCSM polypeptide consensus sequence derived from a comparison of two or more NCSM polypeptide sequences described herein. For example, the present invention includes at least one

15   CD28BP or CTLA-4BP polypeptide consensus sequences derived from a comparison of, respectively, two or more CD28BP or CTLA-4BP polypeptide sequences described herein. A CD28BP polypeptide consensus sequence as used herein refers to a nonnaturally-occurring or recombinant polypeptide that predominantly includes those amino acid residues that are common to all CD28BP polypeptides of the present invention described

20   herein (e.g., full-length and ECD polypeptides and fragments having activities described herein) and that includes, at one or more of those positions wherein there is no amino acid common to all subtypes, an amino acid that predominantly occurs at that position and in no event includes any amino acid residue that is not extant in that position in at least one CD28BP of the invention. A CD28BP polypeptide consensus sequence may have at least

25   one property of a CD28BP polypeptide as described herein (e.g., CD28/CTLA-4 binding affinity ratio at least about equal to greater than that of hB7-1; and/or ability to enhance an immune response, stimulate T cell proliferation or activation).

A CTLA-4BP polypeptide consensus sequence refers to a nonnaturally-occurring or recombinant polypeptide that predominantly includes those amino acid

30   residues which are common to all CTLA-4BP polypeptides of the present invention (e.g., full-length and ECD polypeptides) and that includes, at one or more of those positions wherein there is no amino acid common to all subtypes, an amino acid that predominantly occurs at that position and in no event includes any amino acid residue that is not extant in

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that position in at least one CTLA-4BP of the invention. A CTLA-4BP consensus polypeptide may have at least one property of a CTLA-4BP polypeptide as described herein (e.g., CTLA-4BP/CD28 binding affinity ratio at least about equal to greater than that of hB7-1; suppress an immune response, or inhibit T cell proliferation or activation).

5   An alignment of the amino acid sequence of the full-length parental WT hB7-1 with each R1 and R2 CD28BP amino acid sequence is shown in Figures 2A-2H. An alignment of the amino acid sequence of the full-length parental WT hB7-1 with each R1 and R2 CTLA-4BP amino acid sequence is shown in Figures 3A-3H. Both figures also show the regions of hB7-1 corresponding to the signal peptide, ECD, transmembrane

10   domain, cytoplasmic domain, and mature region (see arrows).) As shown, a number of the CD28BP and CTLA-4BP sequences include two additional amino acid residues in the putative signal sequence, as shown by comparison of these recombinant (chimeric) NCSMs with hB7-1; thus, the ECD for these sequences putatively begins at amino acid residue 37. Of the 7 parental species used for recursive sequence recombination, only the bovine amino

15   acid sequence includes two additional amino acid residues in the putative signal peptide sequence.

In one aspect, the invention provides the CD28BP consensus polypeptide sequence (SEQ ID NO:283) and the CTLA-4BP consensus polypeptide sequence (SEQ ID NO:286) and respective fragments or subsequences thereof that have at least one property of

20   a CD28BP or CTLA-4BP polypeptide as described herein. A subsequence of a CD28BP or CTLA-4 consensus sequence includes a sequence that substantially corresponds (via visual inspection of alignment) to each of the ECD, transmembrane domain, cytoplasmic domain, signal peptide, or mature region of any respective CD28BP or CTLA-4BP polypeptide shown in the alignment in Figures 2A-2H and 3A-3H.

25   The present invention also includes fragments and subsequences of the other CD28BP and CTLA-4BP amino acid sequences shown in Figures 2A-2H and 3A-H, respectively, and nucleic acids encoding such fragments and subsequences. Some such CD28BP and CTLA-4BP amino acid fragments and subsequences have at least one property similar or equivalent (or improved upon) to a CD28BP or CTLA-4BP polypeptide,

30   respectively, as described above.

In particular, the invention includes amino acid fragments or subsequences of the CD28BP or CTLA-4BP shown in Figures 2A-2H and 3A-H, respectively, and nucleic acid sequences encoding such fragments and subsequences, wherein said fragments or

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subsequences comprise at least one of the mature domain, ECD, transmembrane domain, signal peptide, and/or cytoplasmic domain of the CD28BP or CTLA-4BP sequences shown in Figures 2A-2H and 3A-H. These domains may be identified by functional analysis, expression pattern, or comparison by amino acid (or nucleic acid) alignment with a

5 corresponding domain of a WT B7-1 sequence.

For example, a hB7-1 polypeptide (or polynucleotide) sequence is aligned with a fragment or subsequence of the invention, with amino acid (or nucleic acid) residues being aligned at equivalent positions. The numbering of amino acid residues (or nucleic acid residues) in a particular domain, such as the ECD, for a CD28BP or CTLA-4BP

10 fragment or subsequence is based upon the numbering of residues in the corresponding CD28BP or CTLA-4BP polypeptide (or polynucleotide) sequence or, if desired, upon the amino acid numbering in a parental or WT B7-1 polypeptide (or polynucleotide) sequence, such as hB7-1. The amino acids comprising a signal sequence, ECD, mature domain, transmembrane domain, or cytoplasmic domain of a C28BP or CTLA-4BP polypeptide of

15 the invention, or polynucleotide encoding same, can be determined by alignment with a corresponding region of a WT B7-1 (e.g., hB7-1) polypeptide, or polynucleotide encoding the same; positions equivalent to those for the WT B7-1 (Figs. 2A-2H and 3A-3H) can be determined.

The invention also provides at least one fragment of an isolated or

20 recombinant CD28BP polypeptide sequence selected from at least one of SEQ ID NOS:48-68, 174-221, 283-285, and 289-293, wherein the fragment binds or specifically binds with a CD28 and/or CTLA4 receptor and/or induces T cell proliferation or activation in conjunction with stimulation of a T cell receptor (e.g., by antigen) as described herein for CD28BP polypeptides, and provided the fragment itself is not an amino acid fragment

25 known in the art to have such properties.

In addition, the invention provides at least one fragment of an isolated or recombinant CTLA-4BP polypeptide sequence selected from at least one of SEQ ID NOS:69-92, 222-272, and 286-288, wherein the fragment binds or specifically binds with a CD28 and/or CTLA4 receptor and/or inhibits T cell activation or proliferation as described

30 herein for CTLA-4BP polypeptides, and further provided the fragment itself is not an amino acid fragment known in the art to have such properties. Fragments of SEQ ID NOS:93-94 having such properties as described for either of CTLA-4BP or CD28 polypeptides are also included.

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Also provided are polypeptide sequences corresponding to at least one of the following components of any of SEQ ID NOS:48-94, 174-252, 263-272, and/or 283-293: a signal peptide, ECD, transmembrane domain, cytoplasmic domain, or mature region or any combination thereof of such components, such as, e.g., a signal peptide and ECD. A recombinant polypeptide comprising one or more of any of these individual components from one such sequence fused to one or more of these individual components at least one additional sequence is also contemplated in the invention.

Making Polypeptides

Recombinant methods for producing and isolating NCSM polypeptides of the invention are described above. In addition to recombinant production, the polypeptides may be produced by direct peptide synthesis using solid-phase techniques (see, e.g., Stewart et al. (1969) Solid-Phase Peptide Synthesis, WH Freeman Co, San Francisco; Merrifield J. (1963) J Am Chem Soc 85:2149-2154). Peptide synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City, Calif.) in accordance with the instructions provided by the manufacturer. For example, subsequences may be chemically synthesized separately and combined using chemical methods to provide full-length NCSM polypeptides or fragments thereof. Alternatively, such sequences may be ordered from any number of companies which specialize in production of polypeptides. Most commonly, NCSM polypeptides are produced by expressing coding nucleic acids and recovering polypeptides, e.g., as described above.

Methods for producing the polypeptides of the invention are also included. One such method comprises introducing into a population of cells any NCSM nucleic acid described herein, which is operatively linked to a regulatory sequence effective to produce the encoded polypeptide, culturing the cells in a culture medium to produce the polypeptide, and isolating the polypeptide from the cells or from the culture medium. An amount of nucleic acid sufficient to facilitate uptake by the cells (transfection) and/or expression of the NCSM polypeptide is utilized. The culture medium can be any described herein and in the Examples. The nucleic acid is introduced into such cells by any delivery method described herein, including, e.g., injection, gene gun, passive uptake, etc. The NCSM nucleic acid may be part of a vector, such as a recombinant expression vector, including a DNA plasmid vector, or any vector described herein. The nucleic acid or vector comprising a NCSM nucleic acid may be prepared and formulated as described herein, above, and in the

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Examples below. Such a nucleic acid or expression vector may be introduced into a population of cells of a mammal in vivo, or selected cells of the mammal (e.g., tumor cells) may be removed from the mammal and the nucleic acid expression vector introduced ex vivo into the population of such cells in an amount sufficient such that uptake and

5   expression of the encoded polypeptide results. Or, a nucleic acid or vector comprising a NCSM nucleic acid is produced using cultured cells in vitro. In one aspect, the method of producing a NCSM polypeptide comprises introducing into a population of cells a recombinant expression vector comprising any NCSM nucleic acid described herein in an amount and formula such that uptake of the vector and expression of the NCSM polypeptide

10   will result; administering the expression vector into a mammal by any introduction/delivery format described herein; and isolating the polypeptide from the mammal or from a byproduct of the mammal.

<u>Using Polypeptides</u>
<u>Antibodies</u>

15   In another aspect of the invention, a NCSM polypeptide or fragments thereof of the invention is used to produce antibodies which have, e.g., diagnostic, therapeutic, or prophylactic uses, e.g., related to the activity, distribution, and expression of NCSM polypeptides and fragments thereof. Antibodies to NCSM polypeptides or peptide fragments thereof of the invention may be generated by methods well known in the art.

20   Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, humanized, single chain, Fab fragments and fragments produced by a Fab expression library. Antibodies, e.g., those that block receptor binding, are especially preferred for therapeutic and/or prophylactic use.

NCSM polypeptides for antibody induction do not require biological

25   activity; however, the polypeptides or oligopeptides are antigenic. Peptides used to induce specific antibodies may have an amino acid sequence consisting of at least about 10 amino acids, preferably at least about 15 or 20 amino acids or at least about 25 or 30 amino acids.. Short stretches of a NCSM polypeptide may be fused with another protein, such as keyhole limpet hemocyanin, and antibody produced against the chimeric molecule.

30   Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art, and many antibodies are available. See, e.g., <u>Current Protocols in Immunology</u>, John Colligan *et al.*, eds., Vols. I-IV (John Wiley & Sons, Inc., NY, 1991 and 2001 Supplement); and Harlow and Lane (1989)<u> Antibodies: A Laboratory Manual </u>Cold

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Spring Harbor Press, NY; Stites et al. (eds.) Basic and Clinical Immunology (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; and Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) Nature 256:495-497. Other suitable techniques for

5   antibody preparation include selection of libraries of recombinant antibodies in phage or similar vectors. *See*, Huse et al. (1989) Science 246:1275-1281; and Ward et al. (1989) Nature 341:544-546. Specific monoclonal and polyclonal antibodies and antisera will usually bind with a K_D of at least about 0.1 μM, preferably at least about 0.01 μM or better, and most typically and preferably, 0.001 μM or better.

10   Detailed methods for preparation of chimeric (humanized) antibodies can be found in U.S. Patent 5,482,856. Additional details on humanization and other antibody production and engineering techniques can be found in Borrebaeck (ed.) (1995) Antibody Engineering, 2nd Edition Freeman and Company, NY (Borrebaeck); McCafferty et al. (1996) Antibody Engineering, A Practical Approach IRL at Oxford Press, Oxford, England

15   (McCafferty), and Paul (1995) Antibody Engineering Protocols Humana Press, Towata, NJ (Paul).In one useful embodiment, this invention provides for fully humanized antibodies against the NCSM polypeptides of the invention or fragments thereof. Humanized antibodies are especially desirable in applications where the antibodies are used as therapeutics and/or prophylactics in vivo in human patients. Human antibodies consist of

20   characteristically human immunoglobulin sequences. The human antibodies of this invention can be produced in using a wide variety of methods (see, e.g., Larrick et al., U.S. Pat. No. 5,001,065, and Borrebaeck McCafferty and Paul, *supra*, for a review). In one embodiment, the human antibodies of the present invention are produced initially in trioma cells. Genes encoding the antibodies are then cloned and expressed in other cells, such as

25   nonhuman mammalian cells. The general approach for producing human antibodies by trioma technology is described by Ostberg et al. (1983), Hybridoma 2:361-367, Ostberg, U.S. Pat. No. 4,634,664, and Engelman et al., U.S. Pat. No. 4,634,666. The antibody-producing cell lines obtained by this method are called triomas because they are descended from three cells – two human and one mouse. Triomas have been found to produce

30   antibody more stably than ordinary hybridomas made from human cells.

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<u>Sequence Variations</u>
<u>Conservatively Modified Variations</u>

NCSM polypeptides of the present invention include conservatively modified variations of the sequences of any of SEQ ID NOS:48-94, 174-252, 263-272, and

5   283-293 and fragments thereof. Such conservatively modified variations comprise substitutions, additions or deletions that alter, add or delete a single amino acid or a small percentage of amino acids (typically less than about 5%, more typically less than about 4%, 2%, or 1%) in any of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293.

For example, a conservatively modified variation (e.g., a deletion) of the 296

10   amino acid polypeptide identified herein as SEQ ID NO:48 will have a length of about 282 amino acids, preferably about 285 amino acids, more preferably about 288 amino acids, still more preferably about 291 amino acids, and still even more preferably about 294 amino acids or more, corresponding to a deletion of less than about 5%, 4%, 3%, 2%, or 1% of the polypeptide sequence.

15   Another example of a conservatively modified variation (e.g., a "conservatively substituted variation") of the polypeptide identified herein as SEQ ID NO:48 will contain "conservative substitutions," according to the six substitution groups set forth in Table 2 (*supra*), in up to about 15 residues (i.e., less than about 5%) of the 296 amino acid polypeptide.

20   As an example, if four conservative substitutions were localized in the region corresponding to amino acids 69-94 of SEQ ID NO:48, examples of conservatively substituted variations of this region,

QKDSK MVLAI LPGKV QVWPE YKNRTI, would include:

<u>N</u>KDSK MV<u>V</u>AI LPGKV QV<u>F</u>PE YKN<u>K</u>TI and

25   QKD<u>A</u>K MVLAI LPG<u>R</u>V Q<u>M</u>WPE YK<u>Q</u>RTI and the like, where conservative substitutions are underlined.

The NCSM polypeptide sequences of the invention or fragments thereof, including conservatively substituted sequences, can be present as part of larger polypeptide sequences such as occur upon the addition of one or more domains for purification of the

30   protein (e.g., poly-his segments, FLAG tag segments, etc.). These additional functional domains either have little or no effect on the activity of the NCSM portion of the protein, or the additional domains can be removed by post synthesis processing steps such as by treatment with a protease, inclusion of an intein, or the like.

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DEFINING NCSM POLYPEPTIDES BY IMMUNOREACTIVITY

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with a polypeptide encoded by the second nucleic acid. A polypeptide is typically substantially identical to a second polypeptide, e.g., where the two peptides differ only by conservative substitutions.

The phrase "specifically (or selectively) binds," "specifically (or selectively) bound," or "specifically (or selectively) immunoreactive with," when referring to a polypeptide, refers to a binding reaction with an antibody which is determinative of the presence of the polypeptide, or an epitope from the polypeptide, in the presence of a heterogeneous population of polypeptides and other biologics. Specific binding between an antibody or other binding agent and an antigen generally means a binding affinity of at least about 10^5 to 10^6 M^{-1}.

Thus, under designated immunoassay conditions, the specified antibodies bind to a particular polypeptide and do not bind in a significant amount to other polypeptides present in the sample. The antibodies raised against a multivalent antigenic polypeptide will generally bind to the polypeptides from which one or more of the epitopes were obtained. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular polypeptide. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular polypeptide. For example, solid-phase ELISA immunoassays, Western blots, or immunohistochemistry are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York (hereinafter "Harlow and Lane"), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. Typically, a specific or selective reaction is at least twice background signal or noise and more typically 2.5x-5x or more than 10 to 100 times background.

The polypeptides of the invention provide structural features that can be recognized, e.g., in immunological assays. The generation of antisera containing antibodies (for at least one antigen) which specifically binds the polypeptides of the invention, as well as the polypeptides which are bound by such antisera, are a feature of the invention. Preferred binding agents, including antibodies described herein, bind NCSM polypeptides and fragments thereof with affinities of at least about 10^6 to 10^7 M^{-1}, and preferably 10^8 M^{-1}

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to 10^9 M^{-1} or 10^{10} M^{-1}. Conventional hybridoma technology can be used to produce antibodies having affinities of up to about 10^9 M-1. However, new technologies, including phage display and transgenic mice, can be used to achieve higher affinities (e.g., up to at least about 10^{12} M^{-1}). In general, a higher binding affinity is advantageous.

5   The invention includes NCSM polypeptides and fragments thereof that specifically bind to or that are specifically immunoreactive with an antibody or polyclonal antisera generated against at least one immunogen comprising at least one amino acid sequence selected from one or more of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293 or fragments thereof. To eliminate cross-reactivity with other peptides, the antibody or

10   antisera is subtracted with polypeptides encoded by sequences such as, e.g., those represented at GenBank accession numbers A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834, AF203442, AF203443, AF216747,

15   AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950. Where the GenBank sequence corresponds to a nucleic acid, a polypeptide encoded by the nucleic acid is generated and used for antibody/antisera

20   subtraction purposes. Where the nucleic acid corresponds to a non-coding sequence, e.g., a pseudo-gene, an amino acid which corresponds to the reading frame of the nucleic acid is generated (e.g., synthetically), or is minimally modified, e.g., to include a start codon, promoter or the like for recombinant production.

            In one typical format, the immunoassay uses a polyclonal antiserum which

25   was raised against one or more NCSM polypeptides comprising one or more of the sequences corresponding to one or more of SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, or a substantial subsequence or fragment thereof (i.e., comprising at least about 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the amino acids of the full length sequence provided). The full set of potential polypeptide immunogens derived from SEQ

30   ID NOS:48-94, 174-252, 263-272, and 283-293 are collectively referred to herein as "the immunogenic polypeptides." The resulting antisera is optionally selected to have low cross-reactivity against the control, e.g., co-stimulatory homologues and any such cross-reactivity is removed by immunoabsorbtion with one or more of the control polypeptides, prior to use

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of the polyclonal antiserum in the immunoassay. Sequences which are substantially identical to such sequences can also be used, e.g., which are at least about 60%, 70%, 75%, 80%, 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more identical, e.g., as determined using BLAST or the other algorithms described herein and

5   above, e.g., using default parameters.

In another aspect, the invention provides an antibody or antisera produced by administering a NCSM polypeptide of the invention to a mammal, which antibody or antisera specifically binds one or more antigens, the one or more antigens comprising a polypeptide comprising one or more of the amino acid sequences SEQ ID NOS:48-94, 174-

10   252, 263-272, and 283-293, which antibody or antisera does not specifically bind to a polypeptide encoded by one or more of GenBank Nucleotide Accession Nos: A92749, A92750, AA983817, AB026121, AB030650, AB030651, AB038153, AF010465, AF065893, AF065894, AF065895, AF065896, AF079519, AF106824, AF106825, AF106828, AF106829, AF106830, AF106831, AF106832, AF106833, AF106834,

15   AF203442, AF203443, AF216747, AF257653, AH004645, AH008762, AX000904, AX000905, D49843, L12586, L12587, M27533, M83073, M83074, M83075, M83077, NM005191, S74541, S74540, S74695, S74696, U05593, U10925, U19833, U19840, U26832, U33063, U33208, U57755, U88622, X60958, Y08823, and Y09950.

The antisera comprises the serum of a subject that has been immunized

20   against at least one antigen. The antiserum can be monovalent or polyvalent; that is, it can contain antibodies specific for one or more antigenic determinants, depending on whether the subject was immunized with one antigen or a mixture of antigens.

Also included is an antibody or antisera (comprising one or more antibodies) which specifically binds a polypeptide comprising a sequence selected from: SEQ ID

25   NOS:48-94, 174-252, 263-272, and 283-293, wherein the antibody or antisera (comprising antibodies) does not specifically bind to a polypeptide encoded by one or more of GenBank Nucleotide Accession Nos. set forth above.

In order to produce antisera for use in an immunoassay, one or more of the immunogenic polypeptides is produced and purified as described herein. For example,

30   recombinant protein may be produced in a mammalian cell line. An inbred strain of mice (used in this assay because results are more reproducible due to the virtual genetic identity of the mice) is immunized with the immunogenic protein(s) in combination with a standard adjuvant, such as Freund's adjuvant, and a standard mouse immunization protocol (see

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Harlow and Lane, *supra*, for a standard description of antibody generation, immunoassay formats and conditions that can be used to determine specific immunoreactivity). Alternatively, one or more synthetic or recombinant polypeptides derived from the sequences disclosed herein is conjugated to a carrier protein and used as an immunogen.

5   Polyclonal antisera are collected and titered against the immunogenic polypeptide in an immunoassay, for example, a solid phase immunoassay with one or more of the immunogenic proteins immobilized on a solid support. Polyclonal antisera with a titer of 10^6 or greater are selected, pooled and subtracted with the control co-stimulatory polypeptides to produce subtracted pooled titered polyclonal antisera.

10   The subtracted pooled titered polyclonal antisera are tested for cross reactivity against the control polypeptides. Preferably at least two of the immunogenic NCSM polypeptides are used in this determination, preferably in conjunction with at least two of the control polypeptides, to identify antibodies which are specifically bound by the immunogenic polypeptide(s).

15   In this comparative assay, discriminatory binding conditions are determined for the subtracted titered polyclonal antisera which result in at least about a 5-10 fold higher signal to noise ratio for binding of the titered polyclonal antisera to the immunogenic NCSM molecules as compared to binding to any control polypeptides. That is, the stringency of the binding reaction is adjusted by the addition of non-specific competitors

20   such as albumin or non-fat dry milk, or by adjusting salt conditions, temperature, or the like. These binding conditions are used in subsequent assays for determining whether a test polypeptide is specifically bound by the pooled subtracted polyclonal antisera. In particular, test polypeptides which show at least a 2-5x higher signal to noise ratio than the control polypeptides under discriminatory binding conditions, and at least about a ½ signal

25   to noise ratio as compared to the immunogenic polypeptide(s), share substantial structural similarity with the immunogenic polypeptides as compared relative to known B7-1 or related co-stimulatory polypeptides, and are thus NCSM polypeptides of the invention.

In another example, immunoassays in the competitive binding format are used for detection of a test polypeptide. For example, as noted, cross-reacting antibodies

30   are removed from the pooled antisera mixture by immunoabsorption with the control polypeptides. The immunogenic polypeptide(s) are then immobilized to a solid support which is exposed to the subtracted pooled antisera. Test proteins are added to the assay to compete for binding to the pooled subtracted antisera. The ability of the test protein(s) to

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compete for binding to the pooled subtracted antisera as compared to the immobilized protein(s) is compared to the ability of the immunogenic polypeptide(s) added to the assay to compete for binding (the immunogenic polypeptides compete effectively with the immobilized immunogenic polypeptides for binding to the pooled antisera). The percent

5   cross-reactivity for the test proteins is calculated, using standard calculations.

In a parallel assay, the ability of the control proteins to compete for binding to the pooled subtracted antisera is determined as compared to the ability of the immunogenic polypeptide(s) to compete for binding to the antisera. Again, the percent cross-reactivity for the control polypeptides is calculated, using standard calculations.

10   Where the percent cross-reactivity is at least 5-10x as high for the test polypeptides, the test polypeptides are said to specifically bind the pooled subtracted antisera.

In general, the immunoabsorbed and pooled antisera can be used in a competitive binding immunoassay as described herein to compare any test polypeptide to the immunogenic polypeptide(s). In order to make this comparison, the two polypeptides

15   are each assayed at a wide range of concentrations and the amount of each polypeptide required to inhibit 50% of the binding of the subtracted antisera to the immobilized protein is determined using standard techniques. If the amount of the test polypeptide required is less than twice the amount of the immunogenic polypeptide that is required, then the test polypeptide is said to specifically bind to an antibody generated to the immunogenic

20   protein, provided the amount is at least about 5-10x as high as for a control polypeptide.

As a final determination of specificity, the pooled antisera is optionally fully immunoabsorbed with the *immunogenic* polypeptide(s) (rather than any control polypeptides) until little or no binding of the resulting immunogenic polypeptide subtracted pooled antisera to the immunogenic polypeptide(s) used in the immunoabsorbtion is

25   detectable. This fully immunoabsorbed antisera is then tested for reactivity with the test polypeptide. If little or no reactivity is observed (i.e., no more than 2x the signal to noise ratio observed for binding of the fully immunoabsorbed antisera to the immunogenic polypeptide), then the test polypeptide is specifically bound by the antisera elicited by the immunogenic protein.

30   <u>PROLIFERATION/ACTIVATION AND ANTI-PROLIFERATION/INACTIVATION PROPERTIES OF NCSM MOLECULES</u>

The effect of the NCSM polypeptides and fragments thereof was examined on T cells as described in the Examples, *infra*. The results indicate that compositions

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comprising a NCSM polypeptide of the present invention or fragment thereof, or a soluble NSCM-ECD and NCSM-ECD-Ig, can be used in methods of the invention to induce or inhibit proliferation and/or activation of T cells, for example, in conjunction with stimulation of T cell receptor (e.g., by antigen or anti-CD3 Ab). The ability of a NCSM

5   polypeptide or fragment thereof to induce or inhibit T cell proliferation/activation is typically measured against the ability of wild-type B7-1 (such as, e.g., a human, primate, or cow B7-1) to induce or inhibit T cell proliferation or activation, e.g., in conjunction with stimulation of T cell receptor (e.g., by antigen or anti-CD3 Ab). Similarly, a NCSM polynucleotide that encodes such a NCSM polypeptide or fragment thereof, or a soluble

10   NSCM-ECD and NCSM-ECD-Ig, can be used in methods of the invention to induce or inhibit proliferation and/or activation of T cells by using e.g., cells transfected with and expressing or secreting such NCSM molecules. Here, too, the ability of the expressed or secreted NCSM peptide molecule to induce or inhibit T cell proliferation and/or activation is measured in the same manner.

15   Inducing or inhibiting of proliferation/activation of T cells can be performed in vitro (as useful, e.g., in a variety of proliferation assays or in generation of, e.g., tumor-antigen specific T cells that can be administered to cancer patients), or in vivo (as useful, e.g., as a therapeutic and/or prophylactic).

            CD28BPs and CTLA-4BPs of the invention (and fragments thereof) and the

20   nucleic acids encoding such CD28BPs and CTLA-4BPs of the invention (and fragments thereof) are useful in numerous applications, either when used as gene-based therapeutics/vaccines or when administered as, e.g., soluble polypeptides, proteins, or fragments thereof, in the presence or absence of a specific antigen or mixture of antigens.

            Compositions of the present invention can be used to prophylactically or

25   therapeutically treat and thereby prevent, alleviate or ameliorate a variety of conditions where stimulation of T cell proliferation/activation or inhibition of T cell proliferation and/or activation would be beneficial to a patient. Such uses include, but are not limited to, e.g., prophylaxis of infectious disease, therapeutic and prophylactic treatment of a variety of chronic infectious diseases, cancers, allergies, autoimmune diseases, septic shock,

30   prevention and treatment of graft versus host disease, and the like; and the prevention of organ transplant rejection and the like.

            The products of the invention can also be used in gene therapy to reduce immune system recognition of cells expressing a transgene, thus prolonging the longevity of

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the expression of the transgene. Generation of transgenic animals expressing products of the invention optionally can be used as sources of organs for humans (e.g., the organs express a CTLA-4BP of the invention which, on the surface of the organ, down-regulates host T cell responses thus reducing risk of rejection), etc.

5   A desired goal in developing CTLA-4BPs was to create NCSM molecules that specifically signal through CTLA-4 and thus can, e.g., induce tolerance, suppress activated T cells, and induce regulatory T cells. Other routes of modifying immune responses (e.g., nonspecific immunosuppression with cyclosporin A, blockage of APC--T cell interaction with CTLA-4-Ig or with Anti-B7 monoclonal antibodies (mAbs), or

10   blockage of APC--B-cell activation with Anti-CD40L Abs) have drawbacks (e.g., they induce general immunosuppression or they inhibit T cell growth, etc.) and do not achieve the goal of CTLA-4BPs of the invention, namely induction of tolerance of antigen-specific T cells. An example (but not limiting) application of CTLA-4BP in gene therapy is illustrated by, e.g., using a vector encoding a CTLA-4BP and a transgene (alternatively the

15   CTLA-4BP and transgene can be on separate plasmids) which is introduced into a target cell and whose gene products are presented on the same cell surface. The transgene (in context with MCH) interacts with the T cell receptor (TCR) on the T cell while the CTLA-4BP interacts with CTLA-4 on the T cell, all of which leads to an inhibition or reduction of T cell response and a prolonged expression of the transgene.

20   The products (i.e., polypeptides, nucleic acids, and fragments thereof) of the present invention can be useful in such things as vaccine adjuvants (e.g., for genetic vaccines, protein vaccines, attenuated or killed viral vaccines). For example, the nucleic acids of CD28BPs and CTLA-4BPs (or fragments thereof) can be components of genetic vaccines and gene therapy vectors (e.g., DNA vaccines, viral vectors), or they can be

25   expressed in cells of interest (e.g., tumor cells, dendritic cells) which then can be used as vaccines or therapeutics. Additionally, products of the invention can be transfected into tumor cells which, after being rendered unable to proliferate (e.g., by irradiation) then can be used as cell-based vaccines. Alternatively, such transfected cells are lysed and the resulting lysate used as a vaccine. CD28BPs can serve as T cell adjuvants and administered

30   in either as nucleic acids (including, e.g., vectors comprising nucleic acids encoding CD28BPs) or as proteins. Use of a wild-type human B7 gene as a component in a DNA vaccine along with an antigen(s) of interest results in both positive and negative signals to T cells since wild-type human B7 can bind with both CD28 and CTLA-4 on T cells.

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However, DNA vaccines encoding a product of the invention, e.g., CD28BP or fragments thereof, can selectively tailor the T cell response, e.g., CD28BP in a DNA vaccine will result in positive signals to T cells (e.g., signals to induce T cell proliferation/activation). For example, a CD28BP is optionally used in a treatment vaccine for melanoma (in the context of TRP-1, TRP-2 and/or tyrosinase). An illustrative, but not limiting example is: intradermal injection of DNA (antigen) followed by subcutaneous injection of protein (CD28BP) with time periods of, e.g., 2 weeks between treatments for, e.g., 4 cycles. The CD28BP presents low risk of cross-reactivity with wild-type in treatment of life threatening melanoma. Fragments of the CD28BP encoding nucleic acid are optionally used in the procedure. As another illustrative, but not limiting example, CTLA-4BPs of the invention or fragments thereof are optionally used in conjunction with MBP (myelin basic protein) in a treatment vaccine for multiple sclerosis which is given, e.g., as an intramuscular injection every 3 weeks for 6 months or as the condition warrants.

A gene-based vaccine utilizing a NCSM (e.g., a CD28BP or CTLA-4BP) or fragments thereof is optionally comprised of a plasmid encoding both the antigen(s) of interest and the NCSM (e.g., either CD28BP or CTLA-4BP) (alternatively, the antigen(s) of interest is on a separate plasmid from the NCSM gene (e.g., the CD28BP or CTLA-4BP gene(s)). The products of the genes of the plasmid(s) are expressed on the surface of, e.g., an APC. Interaction occurs between the antigen of interest (in the context of MHC) and CD28BP (both on the, e.g., APC) with, respectively, the T cell receptor and CD28 on the T cell which leads to T cell proliferation/activation. Optionally, interaction occurs between the antigen of interest (in the context of MHC) and CTLA-4BP (both on the, e.g., APC) with, respectively, the TCR and CTLA-4 on the T cell which leads to T cell anergy/tolerance.

Another example of CD28BP application is illustrated by inducing specific T cell activation through use of a plasmid encoding a CD28BP or fragments thereof. The plasmid is transfected into a tumor cell (e.g., ex vivo), which is, e.g., irradiated to stop proliferation, and is then used as a vaccine (or optionally a tumor cell lysate, e.g., Melacine ® is used). The tumor antigens are presented (in context with MHC) to the T cell and interact with the TCR. Additionally, the CD28BP expressed on the cell along with the tumor antigen is presented to the T cell and interacts with CD28, thus leading to T cell activation.

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SOLUBLE NCSM POLYPEPTIDES AND NUCLEIC ACIDS

The present invention provides soluble NCSM polypeptides (or fragments thereof) and nucleic acids encoding them. Selected regions (e.g., the ECD, truncated extracellular domain, secreted subsequence of a NCSM polypeptide) or fragments thereof

5   are provided in both polypeptide and nucleic acid format. These soluble molecules are suited for use as prophylactics, therapeutics, and/or diagnostic tools and can be targeted or designed for specific actions and a variety of applications as described herein.

Soluble B7-1 proteins and fragments have been described and characterized. See, e.g., U.S. Pat. No. 6,071,716. Standard procedures for expressing soluble B7-1

10   proteins and fragments thereof, recovering such molecules from culture media, screening and characterizing such molecules for e.g., T cell proliferation or lymphokine production, as described in, e.g., US Pat. No. 6,071,716, can be used and applied to soluble NCSM polypeptides and fragments thereof of the present invention.

A "soluble" NCSM polypeptide, such as a soluble CD28BP or CTLA-4BP of

15   the invention, means a polypeptide comprising an amino acid sequence that corresponds to that of the extracellular domain (ECD) of a NCSM polypeptide or a fragment of said ECD (e.g., a truncated ECD). The soluble NCSM polypeptide typically does not include the amino acid sequences corresponding to the full-length cytoplasmic or transmembrane domain. The amino acid sequence corresponding to the signal peptide or leader, or a

20   fragment thereof, may or may not be included in the soluble NCSM polypeptide. A soluble NSCM polypeptide may further comprise an immunoglobulin (Ig) or Ig fragment, such as, e.g., an Fc portion of an Ig (e.g., IgG) linked to an NCSM ECD or fragment thereof. In one aspect, a soluble NCSM polypeptide comprises a fusion protein comprising an NCSM ECD or fragment thereof and an Ig or fragment thereof, including, e.g., an Fc portion. The Ig

25   may be from a human, primate, or other mammal. A soluble NCSM polypeptide is freely secreted into the medium surrounding a host cell when it is recombinantly produced in the host cell. Nucleic acids encoding any such soluble NCSM polypeptides (or fragments thereof) described above and hereinafter are also an aspect of the invention.

For each NCSM molecule of the invention, a putative ECD polypeptide

30   sequence (or nucleotide sequence encoding said polypeptide sequence) may be determined by alignment of the NCSM ECD polypeptide sequence (or nucleotide sequence encoding same) with an analogous ECD polypeptide sequence (or nucleotide sequence encoding same) of human B7-1 or other mammalian B7-1 (e.g., primate). The putative amino acid

and nucleic acid sequences corresponding to the respective putative signal peptide, transmembrane domain, cytoplasmic domain, and mature region can also be similarly determined for each NCSM molecule of the invention. It is readily understood by one of ordinary in the art that each of these domains/regions of the NCSM polypeptides and

5   polynucleotides determined by such alignment comparison is putative and thus may vary in length by one or more amino acids or nucleic acids, respectively. One of skill can readily confirm such domains/regions by other analyses known in the art, including those used to determine corresponding domains/regions in hB7-1.

The soluble NCSM molecules can show preferential binding to either CTLA-

10   4 or CD28 receptor. Soluble CD28BPs (and fragments thereof) can bind preferentially with CD28 and CTLA-4BPs (and fragments thereof) can bind preferentially with CTLA-4 as compared to the binding of soluble wild-type (WT) human B7-1 to CD28 and CTLA-4. For example, when an antigen is presented (in context with MHC) on the surface of a cell where it interacts with the TCR on a T cell, a soluble CD28BP optionally can interact

15   simultaneously with the T cell through the CD28 molecule, thus leading to T cell proliferation/activation. Conversely, when an antigen is presented (in context with MHC) on the surface of a cell where it interacts with the TCR on a T cell while simultaneously a soluble CTLA-4BP also interacts with the T cell (through the CTLA-4 molecule), T cell anergy/tolerance can result.

20   Optionally, and additionally, the soluble NCSM molecules of the invention, or fragments thereof, can be used as agonists or antagonists of the respective T cell receptors. The soluble CD28BP molecules can optionally act as agonists by stimulating T cell proliferation/activation by binding with CD28 or the soluble CD28BP molecules can optionally act as antagonists by binding with CD28 without stimulating T cell

25   proliferation/activation. Furthermore, soluble CTLA-4BP molecules can optionally act as agonists by binding with CTLA-4 and inhibiting T cell proliferation/activation (e.g., not stimulating T cells) or the soluble CTLA-4BP molecules can act as antagonists by binding with CTLA-4 and not inhibiting T cell proliferation/activation.

These soluble NCSM molecules can be delivered to a subject by a variety of

30   formats. For example, the soluble NCSM molecules can be delivered as polypeptides or proteins (or fragments thereof) or as nucleic acids (or fragments thereof) encoding such polypeptides or proteins.

In one aspect, the invention includes an isolated or recombinant nucleic acid comprising a polynucleotide sequence selected from: (a) a polynucleotide sequence selected from SEQ ID NOS:1-21 and 95-142, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOS:48-68,

5   174-221, 283-285, and 290-293, or a complementary polynucleotide sequence thereof; (c) a polynucleotide sequence which, but for the degeneracy of the genetic code, hybridizes under at least stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b); and (d) a polynucleotide sequence comprising all or a nucleotide fragment of (a), (b), or (c), wherein the nucleotide fragment encodes a soluble polypeptide

10 having a CD28/CTLA-4 binding affinity ratio about equal to or greater than the CD28/CTLA-4 binding affinity ratio of human B7-1 and/or a soluble polypeptide that, in the presence of activated T cells, down-regulates or inhibits a T cell proliferation response compared to the response of soluble hB7-1 (e.g., hB7-1-ECD or hB7-1-ECD-Ig)--that is causes a lower T cell proliferation response that does soluble hB7-1.The present invention

15 also provides fusion proteins, including soluble fusion proteins, comprising a fusion of a protein (such as a CD28BP, CTLA-4BP, B7-1 polypeptide variant, or B7-2 polypeptide variant of the current invention) or fragments thereof with an immunoglobulin (Ig) or portion of an immunoglobulin. The resulting protein-Ig fusions (or immunoadhesions or Fc-fusion proteins) can show improved pharmacokinetics, such as longer half-life in vivo,

20 and/or increased expression. Such fusion proteins can also simplify purification and augment isotype effector functions for specific proteins. See, e.g., Ashkenazi, A. et al. (1997) Curr Op in Immunol 9(2):195-200 for a review of the uses and applications of Ig-protein fusions, which is incorporated by reference in its entirety for all purposes. Examples of Ig protein fusion components, including peptide linkers and Fc regions, that

25 are can be fused to NCSM molecules of the invention, including full-length NCSM molecules and at least one component thereof (e.g., signal peptide, ECD, TM, and/or CD of such NCSM), and B7-1 and B7-2 variants of the invention, are provided in Ashkenazi et al. For example, the invention includes a nucleic acid sequence encoding the signal peptide and ECD of an NCSM described herein can be fused to a nucleotide sequence encoding a linker

30 peptide and/or Fc region and a fusion protein encoded therefrom. The NCSMs of the invention can be fused to many variations of, e.g., immunoglobulins and the NCSM fusions are not limited by, e.g., the type of Ig molecule used. In addition to full length NCSM molecules, fragments of NCSM molecules (e.g., the extracellular domain (ECD) or

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fragments of the ECD) can be fused to Ig molecules. Various sequences, e.g., peptide linker sequences, proteolytic cleavage sites (such as, e.g., Factor Xa cleavage site), etc., can also be incorporated into the NCSM-Ig fusion protein. In some embodiments, the small peptide linker forming the in-frame translational coupling between an NCSM ECD (or hB7-1) and

5   the IgG1 Fc comprised the amino acid sequence valine-threonine (VT) or glycine-valine-threonine (GVT), depending upon the nucleotide sequence compatibility of the 3' codon of the NCSM ECD (see, e.g., Fig. 14B and Example IV below). The length and nature of the amino acid sequence of the peptide linker positioned between the Fc region and ECD is believed important for providing proper contact between the Fc regions of NCSM-ECD

10   monomers or NCSM-ECD-Ig fusion monomers and facilitating or enhancing multimerization of such NCSM-ECD or NCSM-ECD-Ig monomers. In one aspect, e.g., the linker sequence may comprise from at least about one to at least about 11 amino acids; the specific amino acid may include residues GVT, as described above, or other amino acid residues having characteristics that facilitate aggregation of NCSM-ECD or NCSM-ECD-Ig

15   fusion monomers. In another aspect, the invention provides multimers of NCSM-ECD and NCSM-ECD-Ig fusion monomers. Nucleotide sequences encoding all such NCSM-ECCD monomers, and NCSM-Ig and NCSM-ECD-Ig fusion proteins are another aspect of the invention.

       A fusion protein comprising a NCSM polypeptide or fragment thereof fused

20   to human IgG Fc domain is an aspect of the present invention, see, e.g., Example 4, *infra*. The NCSM-Ig fusion proteins have the benefits of being soluble (thus, e.g., expanding their uses as prophylactics and therapeutics) and being stabilized by the Ig portion of the fusion. The soluble NCSM polypeptide-IgG fusion proteins of the present invention are suited for use as prophylactics and/or therapeutics since they can be targeted or tailored for specific

25   actions and a variety of applications.

       The NCSM-Ig fusion proteins and polypeptides of the invention can be used for, e.g., similar applications (including, e.g., therapeutic, prophylactic, and diagnostic applications described herein) as the NCSM proteins and polypeptides of the invention (as indicated throughout). These Ig-fusion proteins of the invention also show preferential

30   binding to either CTLA-4 or CD28. CD28BP-Ig fusions (and fragments thereof) bind preferentially with CD28 and CTLA-4BP-Ig fusions (and fragments thereof) bind preferentially with CTLA-4 as compared to the binding of human B7-1 to CD28 and CTLA-4. For example, when an antigen is presented (in context with MHC) on the surface

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of a cell where it interacts with the TCR on a T cell, a soluble CD28BP-Ig fusion protein optionally can interact simultaneously with the T cell through the CD28 molecule, thus leading to T cell proliferation/activation. Conversely, when an antigen is presented (in context with MHC) on the surface of a cell where it interacts with the TCR on a T cell while

5 simultaneously a soluble CTLA-4BP-Ig fusion protein also interacts with the T cell (through the CTLA-4 molecule), T cell anergy/tolerance can result.

Optionally, the soluble NCSM-Ig fusion proteins (e.g., CD28BP-Ig and CTLA-4BP-Ig), or fragments thereof, or proteins (or protein fusions) based on the NCSMs (e.g., CD28BPs or CTLA-4BPs) are used as agonists or antagonists of the respective

10 receptors on the T cell. For example, CD28BP or CD28BP-Ig fusions acting as agonists by stimulating T cell proliferation/activation through binding to CD28 or as antagonists by binding to CD28 without inducing T cell proliferation/activation. Alternatively, CTLA-4 or CTLA-4BP-Ig fusions acting as agonists by inhibiting (or not stimulating) T cell proliferation/activation through binding to CTLA-4 or as antagonists by binding to CTLA-4

15 without inhibiting T cell proliferation/activation.

In one aspect, the IgG Fc hinge, CH2 and CH3 domains are modified or evolved using a recursive sequence recombination method, such as DNA shuffling, or another diversity generation method to produce a library of recombinant IgG Fc hinge CH2 and CH3 domains from a group of selected parental IgG Fc sequences, and then screening

20 the library by appropriate screening procedures to identity a chimeric IgG Fc comprising at least one recombined Fc constant domain exhibiting reduced binding to at least one Fcγ receptor – e.g., FcγRII and FcγRIII. Specifically, the library of recombinant (shuffled) IgG molecules are digested with BsteII and EcoRI, and ligated into a BstEII – EcoRI digested plasmid comprising at least one NCSM nucleic acid sequence to produce a non- or reduced-

25 FcγR binding NCSM-Ig fusion protein molecules. Supernatants from 293 cells transiently transfected with such expression plasmids are incubated with 293 cells expressing either FcγRII or FcγRIII, and binding affinity of the expressed recombinant clones is determined by FACS analysis methods using a NCSM-specific mAb conjugated with FITC. IgG Fc variants exhibiting reduced or no binding to one or more FcγR are used as fusion partners

30 with one or more specific NCSMs of the present invention and are useful in the applications described above.

In addition to fusion with Ig sequences or regions, the fusion proteins of the invention can include any protein sequence in combination with the NCSM molecule, or

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fragment thereof. The invention also includes the nucleic acid sequence encoding any such fusion polypeptide. For example, a NCSM polypeptide of the invention or fragments thereof can be fused with polypeptide sequences which, e.g., enable sorting of the fusion proteins, e.g., fluorescence indicator molecules. Alternatively, the NCSM polypeptides or

5   fragments thereof can be incorporated into fusion proteins that enable, e.g., targeting of the fusions to specific cell types or cells.

The fusion proteins of the present invention can be delivered to a subject by a variety of formats, including, e.g., as a polypeptide or protein, or as a nucleic acid encoding such polypeptide or protein as described in detail herein.

10   THERAPEUTIC AND PROPHYLACTIC TREATMENT METHODS

The NCSM polynucleotides and polypeptides of the invention have properties that are of beneficial use in a variety of application, including, e.g., protein- and DNA-based vaccinations and in prophylactic and therapeutic disease treatments where manipulation of an immune response (e.g., inducing or suppressing), T cell activation or

15   proliferation, and/or cytokine production is desirable.

In one aspect, the present invention includes methods of therapeutically or prophylactically treating a disease or disorder by administering, in vivo or ex vivo, one or more nucleic acids or fragments thereof or polypeptides or fragments thereof of the invention described above (or compositions, vectors, or transduced cells comprising a

20   pharmaceutically acceptable excipient and one or more such nucleic acids or polypeptides) to a subject or to a population of cells of the subject, including, e.g., a mammal, including, e.g., a human, primate, monkey, orangutan, baboon, mouse, pig, cow, cat, goat, rabbit, rat, guinea pig, hamster, horse, sheep; or a non-mammalian vertebrate such as a bird (e.g., a chicken or duck) or a fish, or invertebrate.

25   In one aspect of the invention, in ex vivo methods, one or more cells or a population of cells of interest of the subject (e.g., tumor cells, tumor tissue sample, organ cells, blood cells, cells of the skin, lung, heart, muscle, brain, mucosae, liver, intestine, spleen, stomach, lymphatic system, cervix, vagina, prostate, mouth, tongue, etc.) are obtained or removed from the subject and contacted with an amount of a polypeptide of the

30   invention that is effective in prophylactically or therapeutically treating a disease, disorder, or other condition. The contacted cells are then returned or delivered to the subject to the site from which they were obtained or to another site (e.g., including those defined above)

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of interest in the subject to be treated. If desired, the contacted cells may be grafted onto a tissue, organ, or system site (including all described above) of interest in the subject using standard and well-known grafting techniques or, e.g., delivered to the blood or lymph system using standard delivery or transfusion techniques.

5   The CD28BP polypeptides of the invention and/or nucleic acids of the invention can be used in methods to activate T cells ex vivo by, e.g., obtaining or removing T cells from a subject (e.g., mammal, such as a human) and administering to the subject a sufficient amount of one or more polypeptides of the invention to activate effectively the T cells (or administering a sufficient amount of one or more nucleic acids of the invention

10   with a promoter such that uptake of the nucleic acid into one or more such T cells occurs and sufficient expression of the nucleic acid results to produce an amount of a polypeptide effective to activate said T cells. The activated T cells are then returned to the subject. T cells can be obtained or isolated from the subject by a variety of methods known in the art, including, e.g., by deriving T cells from peripheral blood of the subject or obtaining T cells

15   directly from a tumor of the subject.

          The CD28BP polypeptides of the invention and/or nucleic acids of the invention can be used to activate T cells ex vivo by, e.g., obtaining or removing cells (e.g., antigen presenting cells) from a subject (e.g., a mammal, such as a human) and administering to the removed cells a sufficient amount of one or more polypeptides of the

20   invention to activate effectively T cells once the removed cells are returned to the subject (or administering a sufficient amount of one or more nucleic acids of the invention with a promoter such that uptake of the nucleic acid into one or more removed cells occurs and sufficient expression of the nucleic acid results to produce an amount of a polypeptide effective to activate T cells upon return of the removed cells to the subject).

25   The CTLA-4BP polypeptides of the invention and/or nucleic acids encoding polypeptides of the invention are useful in inhibiting T cell response (e.g., inhibiting T cell activation or proliferation) in a subject to which at least one at the polypeptides or nucleic acids of the invention is administered. In another aspect, the CTLA-4BP polypeptides of the invention and/or nucleic acids encoding polypeptides of the invention modulate T cell

30   activation without completely inhibiting T cell proliferation following administration. In another aspect, the CTLA-4BP polypeptides of the invention and/or nucleic acids encoding polypeptides of the invention modulate T cell activation in a subject following administration, but do not induce proliferation of purified T cells activated by soluble

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monoclonal antibodies (e.g., anti-CD3 monoclonal antibodies that bind T cell receptor (TCR) on a T cell).

The invention also provides in vivo methods in which at least one cell or a population of cells of interest of the subject are contacted directly or indirectly with a

5   sufficient amount of a NCSM polypeptide of the invention effective in prophylactically or therapeutically treating a disease, disorder, or other condition. In direct (e.g., local) contact or administration formats, the polypeptide is typically administered or transferred directly (e.g., locally) to the cells to be treated or to the tissue site of interest (e.g., tumor cells, tumor tissue sample, organ cells, blood cells, cells of the skin, lung, heart, muscle, brain, mucosae,

10   liver, intestine, spleen, stomach, lymphatic system, cervix, vagina, prostate, mouth, tongue, etc.) by any of a variety of formats, including topical administration, injection (e.g., using a needle or syringe), or vaccine or gene gun delivery, or pushing into a tissue, organ, or skin site.

The NCSM molecule can be delivered by a variety of routes, e.g.,

15   intramuscularly, intradermally, subdermally, subcutaneously, orally, intraperitoneally, intrathecally, intravenously, mucosally, systemically, parenterally, via inhalation, or placed within a cavity of the body (including, e.g., during surgery), or by inhalation or vaginal or rectal administration.

In *in vivo* and *ex vivo* indirect contact/administration formats, the NCSM

20   polypeptide is typically administered or transferred indirectly to the cells to be treated or to the tissue site of interest, including those described above (such as, e.g., skin cells, organ systems, lymphatic system, or blood cell system, etc.), by contacting or administering the NCSM polypeptide of the invention directly to one or more cells or population of cells from which treatment can be facilitated. For example, tumor cells within the body of the subject

25   can be treated by contacting cells of the blood or lymphatic system, skin, or an organ with a sufficient amount of the polypeptide such that delivery of the polypeptide to the site of interest (e.g., tissue, organ, or cells of interest or blood or lymphatic system within the body) occurs and effective prophylactic or therapeutic treatment results. Such contact, administration, or transfer is typically made by using one or more of the routes or modes of

30   administration described above.

In another aspect, the invention provides ex vivo methods in which one or more cells of interest or a population of cells of interest of the subject (e.g., tumor cells, tumor tissue sample, organ cells, blood cells, cells of the skin, lung, heart, muscle, brain,

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mucosae, liver, intestine, spleen, stomach, lymphatic system, cervix, vagina, prostate, mouth, tongue, etc.) are obtained or removed from the subject and transformed by contacting said one or more cells or population of cells with a polynucleotide construct comprising a target nucleic acid sequence of the invention or fragments thereof, that

5   encodes a biologically active polypeptide of interest (e.g., a polypeptide of the invention) that is effective in prophylactically or therapeutically treating the disease, disorder, or other condition. The one or more cells or population of cells is contacted with a sufficient amount of the polynucleotide construct and a promoter controlling expression of said nucleic acid sequence such that uptake of the polynucleotide construct (and promoter) into the cell(s)

10   occurs and sufficient expression of the target nucleic acid sequence of the invention results to produce an amount of the biologically active polypeptide effective to prophylactically or therapeutically treat the disease, disorder, or condition. The polynucleotide construct may include a promoter sequence (e.g., WT, recombinant, or chimeric CMV promoter sequence) that controls expression of a NCSM nucleic acid sequence of the invention and/or, if

15   desired, one or more additional nucleotide sequences encoding at least one of another NCSM polypeptide, a cytokine, an adjuvant, or a co-stimulatory molecule, or other polypeptide of interest.

Following transfection, the transformed cells are returned, delivered, or transferred to the subject to the tissue site or system from which they were obtained or to

20   another site (e.g., tumor cells, tumor tissue sample, organ cells, blood cells, cells of the skin, lung, heart, muscle, brain, mucosae, liver, intestine, spleen, stomach, lymphatic system, cervix, vagina, prostate, mouth, tongue, etc.) to be treated in the subject. If desired, the cells may be grafted onto a tissue, skin, organ, or body system of interest in the subject using standard and well-known grafting techniques or delivered to the blood or lymphatic system

25   using standard delivery or transfusion techniques. Such delivery, administration, or transfer of transformed cells is typically made by using one or more of the routes or modes of administration described above. Expression of the target nucleic acid occurs naturally or can be induced (as described in greater detail below) and an amount of the encoded polypeptide is expressed sufficient and effective to treat the disease or condition at the site

30   or tissue system.

In another aspect, the invention provides in vivo methods in which one or more cells of interest or a population of cells of the subject (e.g., including those cells and cell(s) systems and subjects described above) are transformed in the body of the subject by

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contacting the cell(s) or population of cells with (or administering or transferring to the cell(s) or population of cells using one or more of the routes or modes of administration described above) a polynucleotide construct comprising a nucleic acid sequence of the invention that encodes a biologically active polypeptide of interest (e.g., a polypeptide of

5   the invention) that is effective in prophylactically or therapeutically treating the disease, disorder, or other condition.

The polynucleotide construct can be directly administered or transferred to cell(s) exhibiting or having the disease or disorder (e.g., by direct contact using one or more of the routes or modes of administration described above). Alternatively, the

10   polynucleotide construct can be indirectly administered or transferred to cell(s) exhibiting or having the disease or disorder by first directly contacting non-diseased cell(s) or other diseased cells using one or more of the routes or modes of administration described above with a sufficient amount of the polynucleotide construct comprising the nucleic acid sequence encoding the biologically active polypeptide, and a promoter controlling

15   expression of the nucleic acid sequence, such that uptake of the polynucleotide construct (and promoter) into the cell(s) occurs and sufficient expression of the nucleic acid sequence of the invention results to produce an amount of the biologically active polypeptide effective to prophylactically or therapeutically treat the disease or disorder, and whereby the polynucleotide construct or the resulting expressed polypeptide is transferred naturally or

20   automatically from the initial delivery site, system, tissue or organ of the subject's body to the diseased site, tissue, organ or system of the subject's body (e.g., via the blood or lymphatic system). Expression of the target nucleic acid occurs naturally or can be induced (as described in greater detail below) such that an amount of the encoded polypeptide expressed is sufficient and effective to treat the disease or condition at the site or tissue

25   system. The polynucleotide construct may include a promoter sequence (e.g., wild-type, recombinant or chimeric CMV promoter sequence) that controls expression of the nucleic acid sequence and/or, if desired, one or more additional nucleotide sequences encoding at least one of another NCSM polypeptide, a cytokine, an adjuvant, or a co-stimulatory molecule, or other polypeptide of interest.

30   In one aspect, tumor cells of a patient are transfected with a DNA plasmid vector encoding a NCSM polypeptide of interest (e.g., CD28BP) to facilitate an improved immune response, (e.g., enhanced T cell response or increased antibody titer). The tumor cells may be removed from the patient and transfected ex vivo, and then re-delivered to the

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patient, preferably at the tumor site. Alternatively, the tumor cells of a tumor are transfected in vivo, by delivering a DNA plasmid encoding a NCSM polypeptide of interest (e.g., CD28BP). In either case, the immune response can be measured by measuring T cell proliferation using methods described herein or antibody levels using standard protocols. In another aspect, a DNA plasmid encoding a soluble NCSM-ECD or soluble NCSM-ECD-Ig is administered to a patient by any means described herein, including systemically, subcutaneously, i.m., intradermally, etc. and the like, via a needle or gene gun or other introduction mechanism described herein; if desired, the plasmid is introduced directly into cells of a tumor or tumor-related cells of the patient.

In yet another aspect, a soluble NCSM-ECD polypeptide or soluble NCSM-ECD-Ig fusion protein is administered to a patient by any means described herein, including systemically, subcutaneously, i.m., intradermally, etc. and the like, via a needle or gene gun or other introduction mechanism described herein; if desired, the polypeptide or fusion protein is introduced directly into cells of a tumor or tumor-related cells of the patient. The soluble NCSM can be administered in conjunction with an antigen (either simultaneously or consecutively) as part of a vaccine protocol.

The NCSM polypeptides of the invention and the NCSM polynucleotides encoding them are also useful as vaccine adjuvants in vaccine applications as discussed herein and for diagnostic purposes, as for in vitro applications for testing and diagnosing such diseases. For example, a polynucleotide encoding a NCSM of the invention, (e.g., CD28BP) or an NCSM polypeptide (or fragment thereof, e.g., ECD, or fusion protein) can serve as an adjuvant to a DNA vaccine or protein vaccine by enhancing immune-stimulating properties of the antigen encoded by the DNA vaccine or the protein antigen itself, respectively. In any of these formats, the NCSM molecule that results may non-specifically enhance the immune response of the subject to an antigen.

In each of the in vivo and ex vivo treatment methods as described above, a composition comprising an excipient and the NCSM polypeptide or nucleic acid of the invention can be administered or delivered. In one aspect, a composition comprising a pharmaceutically acceptable excipient (e.g., PBS) and a NCSM polypeptide or nucleic acid of the invention is administered or delivered to the subject as described above in an amount effective to treat the disease or disorder.

In another aspect, in each in vivo and ex vivo treatment method described above, the amount of polynucleotide administered to the cell(s) or subject can be an amount

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sufficient that uptake of said polynucleotide into one or more cells of the subject occurs and sufficient expression of said nucleic acid sequence results to produce an amount of a biologically active NCSM polypeptide (e.g., ECD) effective to enhance an immune response in the subject, including an immune response induced by an immunogen (e.g.,

5 antigen). In another aspect, for each such method, the amount of polypeptide administered to cell(s) or subject can be an amount sufficient to enhance an immune response in the subject, including that induced by an immunogen (e.g., antigen).

In yet another aspect, in each in vivo and ex vivo treatment method described above, the amount of polynucleotide administered to the cell(s) or subject can be an amount

10 sufficient that uptake of said polynucleotide into one or more cells of the subject occurs and sufficient expression of said nucleic acid sequence results to produce an amount of a biologically active polypeptide effective to produce a tolerance or anergy response in the subject. In another aspect, for each such method, the amount of polypeptide administered to cell(s) or subject can be an amount sufficient to produce a tolerance or anergy response in

15 the subject.

The amount of DNA plasmid for use in such methods where administration is by injection is from about 50 micrograms (ug) to 5 mg, usually about 100 ug to about 2.5 mg, typically about 500 ug to 2 mg or about 800 ug to about 1.5 mg, and often about 1 mg. The amount of DNA plasmid for use in these methods where administration is via a gene

20 gun, e.g., is from about 100 to 1000 times less; thus, for each range given above for DNA plasmid administration via injection, the range for DNA plasmid administration via gene gun is about 100 to 1000 times less. For example, for gene gun delivery, the amount of DNA plasmid corresponding to the first range above is from about 50×10^{-8} g to 5×10^{-5} g (100 times less) or from about 50×10^{-9} to about 5×10^{-6} g. DNA plasmid amounts can be

25 readily adjusted by those of ordinary skill in the art based upon responses in animal models obtained using the DNA plasmid vector encoding WT hB7-1 and/or antigen or based upon known DNA vaccination studies using plasmid vectors encoding a mammalian B7-1, such as WT hB7-1. Such amounts of DNA plasmid can be used, if desired, in the method in Example VI.

30 In yet another aspect, in an in vivo or in vivo treatment method in which a polynucleotide construct (or composition comprising a polynucleotide construct) is used to deliver a physiologically active polypeptide to a subject, the expression of the polynucleotide construct can be induced by using an inducible on- and off-gene expression

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system. Examples of such on- and off-gene expression systems include the Tet-On™ Gene Expression System and Tet-Off™ Gene Expression System (see, e.g., Clontech Catalog 2000, pg. 110-111 for a detailed description of each such system), respectively. Other controllable or inducible on- and off-gene expression systems are known to those of

5   ordinary skill in the art. With such system, expression of the target nucleic of the polynucleotide construct can be regulated in a precise, reversible, and quantitative manner. Gene expression of the target nucleic acid can be induced, for example, after the stable transfected cells containing the polynucleotide construct comprising the target nucleic acid are delivered or transferred to or made to contact the tissue site, organ or system of interest.

10   Such systems are of particular benefit in treatment methods and formats in which it is advantageous to delay or precisely control expression of the target nucleic acid (e.g., to allow time for completion of surgery and/or healing following surgery; to allow time for the polynucleotide construct comprising the target nucleic acid to reach the site, cells, system, or tissue to be treated; to allow time for the graft containing cells transformed with the

15   construct to become incorporated into the tissue or organ onto or into which it has been spliced or attached, etc.).

The present invention also provides a therapeutic method of activating or enhancing a T cell response in a subject suffering from a cancer, such as, e.g., where the subject has a tumor. The method comprises administering to the subject a composition that

20   comprises a nucleotide sequence that encodes a soluble NCSM polypeptide and an excipient, wherein the NCSM polypeptide is expressed by the tumor cells or the tumor-related cells, and the T cell response is activated or enhanced against the tumor. The composition may be a pharmaceutical composition, and the excipient may be a pharmaceutically acceptable excipient. The pharmaceutical composition may comprise a

25   nucleotide sequence encoding a soluble NCSM polypeptide (or fragment thereof having at least one NCSM property) and a pharmaceutically acceptable excipient. Such nucleotide sequence may be incorporated in a vector and may be operably linked to a promoter to facilitate expression.

In one embodiment, the composition comprising a nucleotide sequence encoding a soluble NCSM polypeptide (or fragment thereof having at least one NCSM

30   property) and an excipient is administered to the subject by i.d., i.m. or, e.g., direct injection or via gene gun or other vaccine delivery device. The composition may be introduced or administered by a variety of routes, including, direct administration to the tumor or tumor

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site, if known, or administration systemically to the subject by direct injection or gene gun or the like. A sufficient amount of the composition is delivered such that transfection of the subject's tumor cells with the NCSM-polypeptide-encoding nucleotide sequence occurs and a T cell response or activation results. As described above, a DNA plasmid expression

5   vector comprising the nucleotide sequence may be delivered as "naked" DNA or may be formulated with other components (e.g., calcium phosphate, lipids, etc.) to facilitate transfection. Exemplary amounts of the total DNA (e.g., in milligrams) (for the NCSM polynucleotide and vector) suggested for such treatment are described herein and in the Examples below. The amount of DNA plasmid may be a therapeutically effective amount

10   to inhibit further growth of the tumor or kill the tumor. One of skill in the art can also determine a therapeutically effective DNA plasmid vector amounts based on known clinical studies to treat cancers using gene therapy or DNA vaccination methods and WT hB7-1 and mammalian models.

In another aspect, tumor cells are obtained from the subject (or alternatively

15   allogeneic tumor cells are used). The tumor cells are transfected using techniques described herein) with a sufficient amount of an expression vector, such as e.g., a pMaxVax vector, described in the Example V below, that comprises a NCSM polynucleotide encoding a NCSM polypeptide (full-length) or soluble NCSM-ECD polypeptide (or NCSM-ECD-Ig fusion protein) such that expression results, and in the case of soluble polypeptides, the

20   soluble polypeptides are secreted.

Genetic Vectors

Gene therapy and genetic vaccine vectors are useful for treating and/or preventing various diseases and other conditions. The following discussion focuses on the on the use of vectors because gene therapy and genetic vaccine method typically employ

25   vectors, but persons of skill in the art appreciate that the nucleic acids of the invention can, depending on the particular application, be employed in the absence of vector sequences. Accordingly, references in the following discussion to vectors should be understood as also relating to nucleic acids of the invention that lack vector sequences. The invention includes vectors comprising one or more NCSM nucleic acids of the invention, including nucleic

30   acids encoding B7-1 and B7-2 polypeptide variants as described herein, including, e.g., variants of human, primate, and bovine B7-1 and B7-2.

Vectors can be delivered to a subject to induce an immune response or other therapeutic or prophylactic response. Suitable subjects include, but are not limited to, a

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mammal, including, e.g., a human, primate, monkey, orangutan, baboon, mouse, pig, cow, cat, goat, rabbit, rat, guinea pig, hamster, horse, sheep; or a non-mammalian vertebrate such as a bird (e.g., a chicken or duck) or a fish, or invertebrate.

Vectors can be delivered *in vivo* by administration to an individual patient, typically by local (direct) administration or by systemic administration (*e.g.*, intravenous, intraperitoneal, intramuscular, subdermal, intracranial, anal, vaginal, oral, mucosal, inhalation, systemic, parenteral, buccal route or they can be inhaled) or they can be administered by topical application. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (*e.g.*, lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

In local (direct) administration formats, the nucleic acid or vector is typically administered or transferred directly to the cells to be treated or to the tissue site of interest (e.g., tumor cells, tumor tissue sample, organ cells, blood cells, cells of the skin, lung, heart, muscle, brain, mucosae, liver, intestine, spleen, stomach, lymphatic system, cervix, vagina, prostate, mouth, tongue, etc.) by any of a variety of formats, including topical administration, injection (e.g., by using a needle or syringe), or vaccine or gene gun delivery, pushing into a tissue, organ, or skin site. For standard gene gun administration, the vector or nucleic acid of interest is precipitated onto the surface of microscopic metal beads. The microprojectiles are accelerated with a shock wave or expanding helium gas, and penetrate tissues to a depth of several cell layers. For example, the AccelTM Gene Delivery Device manufactured by Agacetus, Inc. Middleton WI is suitable for use in this embodiment. The nucleic acid or vector can be delivered, for example, intramuscularly, intradermally, subdermally, subcutaneously, orally, intraperitoneally, intrathecally, intravenously, mucosally, systemically, parenterally, via inhalation, or placed within a cavity of the body (including, e.g., during surgery), or by inhalation or vaginal or rectal administration.

In in vivo indirect contact/administration formats, the nucleic acid or vector is typically administered or transferred indirectly to the cells to be treated or to the tissue site of interest, including those described above (such as, e.g., skin cells, organ systems, lymphatic system, or blood cell system, etc.), by contacting or administering the nucleic acid or vector of the invention directly to one or more cells or population of cells from

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which treatment can be facilitated. For example, tumor cells within the body of the subject can be treated by contacting cells of the blood or lymphatic system, skin, or an organ with a sufficient amount of the polypeptide such that delivery of the nucleic acid or vector to the site of interest (e.g., tissue, organ, or cells of interest or blood or lymphatic system within the body) occurs and effective prophylactic or therapeutic treatment results. Such contact, administration, or transfer is typically made by using one or more of the routes or modes of administration described above.

A large number of delivery methods are well known to those of skill in the art. Such methods include, for example liposome-based gene delivery (Debs and Zhu (1993) WO 93/24640; Mannino and Gould-Fogerite (1988) *BioTechniques* 6(7):682-691; Rose U.S. Pat No. 5,279,833; Brigham (1991) WO 91/06309; and Felgner *et al.* (1987) *Proc. Natl Acad. Sci. USA* 84:7413-7414), as well as use of viral vectors (*e.g.*, adenoviral (*see, e.g.*, Berns *et al.* (1995) *Ann. NY Acad. Sci.* 772:95-104; Ali *et al.* (1994) *Gene Ther.* 1:367-384; and Haddada *et al.* (1995) *Curr. Top. Microbiol. Immunol.* 199 (Pt 3):297-306 for review), papillomaviral, retroviral (*see, e.g.*, Buchscher *et al.* (1992) *J. Virol.* 66(5) 2731-2739; Johann *et al.* (1992) *J. Virol.* 66 (5):1635-1640 (1992); Sommerfelt *et al.*, (1990) *Virol.* 176:58-59; Wilson *et al.* (1989) *J. Virol.* 63:2374-2378; Miller et al., *J. Virol.* 65:2220-2224 (1991); Wong-Staal *et al.*, PCT/US94/05700, and Rosenburg and Fauci (1993) in *Fundamental Immunology, Third Edition* Paul (ed) Raven Press, Ltd., New York and the references therein, and Yu *et al.*, *Gene Therapy* (1994) *supra.*), and adeno-associated viral vectors (*see*, West *et al.* (1987) *Virology* 160:38-47; Carter *et al.* (1989) U.S. Patent No. 4,797,368; Carter *et al.* WO 93/24641 (1993); Kotin (1994) *Human Gene Therapy* 5:793-801; Muzyczka (1994) *J. Clin. Invst.* 94:1351 and Samulski (*supra*) for an overview of AAV vectors; *see also*, Lebkowski, U.S. Pat. No. 5,173,414; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 5(11):3251-3260; Tratschin, *et al.* (1984) *Mol. Cell. Biol.*, 4:2072-2081; Hermonat and Muzyczka (1984) *Proc. Natl Acad. Sci. USA*, 81:6466-6470; McLaughlin *et al.* (1988) and Samulski *et al.* (1989) *J. Virol.*, 63:03822-3828), and the like.

"Naked" DNA and/or RNA that comprises a genetic vaccine can also be introduced directly into a tissue, such as muscle, by injection using a needle or other similar device. *See, e.g.*, US Pat. No. 5,580,859. Other methods such as "biolistic" or particle-mediated transformation (*see, e.g.*, Sanford *et al.*, USPN 4,945,050; USPN 5,036,006) are also suitable for introduction of genetic vaccines into cells of a mammal according to the invention. These methods are useful not only for *in vivo* introduction of DNA into a

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subject, such as a mammal, but also for *ex vivo* modification of cells for reintroduction into a mammal. DNA is conveniently introduced directly into the cells of a mammal or other subject using, e.g., injection, such as via a needle, or a "gene gun." As for other methods of delivering genetic vaccines, if necessary, vaccine administration is repeated in order to

5   maintain the desired level of immunomodulation, such as the level or response of T cell activation or T cell proliferation, or antibody titer level. Alternatively, nucleotides can be impressed into the skin of the subject.

        Gene therapy and genetic vaccine vectors (*e.g.*, DNA, plasmids, expression vectors, adenoviruses, liposomes, papillomaviruses, retroviruses, *etc.*) comprising at least

10   one NCSM sequence can be administered directly to the subject (usually a mammal) for transduction of cells *in vivo* or *ex vivo*. The vectors can be formulated as pharmaceutical compositions for administration in any suitable manner, including parenteral (*e.g.*, subcutaneous, intramuscular, intradermal, or intravenous), inhalation, mucosal, topical, oral, rectal, vaginal, intrathecal, buccal (*e.g.*, sublingual), or local administration, such as by

15   aerosol or transdermally, for immunotherapeutic or other prophylactic and/or therapeutic treatment. Pretreatment of skin, for example, by use of hair-removing agents, may be useful in transdermal delivery. Suitable methods of administering such packaged nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more

20   immediate and more effective reaction than another route.

        Further, the vectors of this invention comprising at least one nucleotide sequence encoding at least one NCSM (and, if desired, further comprising a nucleotide sequence encoding antigen or other co-stimulatory molecule co-expressed on the same vector) can be used to prophylactically or therapeutically treat or supplement such treatment

25   of other immunological disorders and diseases or enhance protection against disorders, diseases , and antigens (including WT and recombinant antigens), e.g., in protein vaccines and DNA vaccines, including, but not limited to, e.g., allergy/asthma, neurological, organ transplantation (e.g., graft versus host disease, and autoimmune diseases), malignant diseases, chronic infectious diseases, including, but not limited to, e.g., viral infectious

30   diseases, such as those associated with, but not limited to, e.g., alpha viruses, hepatitis viruses, e.g., hepatitis B virus (HBV), herpes simplex virus (HSV), hepatitis C virus (HCV), HIV, human papilloma virus (HPV), malaria, Venezuelan equine encephalitis (VEE), Western equine encephalitis (WEE), Japanese encephalitis virus, Eastern equine

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encephalitis, and the like, and bacterial infectious diseases, such as, e.g., but not limited to, e.g., Lyme disease, tuberculosis, and chlamydia infections; and other diseases and disorders described herein.

If desired, a separate vector comprising a nucleotide sequence encoding an antigen or other co-stimulatory molecule can be delivered simultaneously with a vector comprising a NCSM sequence of the invention.

Compositions and Formulations

The present invention also includes compositions of any NSCM nucleic acid or NCSM polypeptide of the invention, including B7-1 and B7-2 variant nucleic acids and polypeptide variants, including, e.g., nucleic acid variants and polypeptide variants of human, primate, and bovine B7-1 and B7-2. The invention also includes compositions comprising one or more vectors or cells (or a population of cells) comprising any such polypeptide or nucleic acid of the invention. In one aspect, the invention provides therapeutic and/or prophylactic compositions comprising at least one NCSM polypeptide (or fragment thereof) or nucleic acid (or fragment thereof) of the invention, or vectors, transduced cells, or vaccines comprising at least one NCSM nucleic acid or polypeptide (or fragment) of the invention. Such compositions optionally are tested in appropriate in vitro and in vivo animal models of disease, to confirm efficacy, tissue metabolism, and to estimate dosages, according to methods well known in the art. In particular, dosages for therapeutic and prophylactic methods for treating or preventing a disease or condition can be determined by activity comparison of the NCSM molecules to other known therapeutics using similar compositions in a relevant assay and mammalian model, including as described below.

Administration optionally is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. See, *supra*. The NCSM polypeptides and polynucleotides, and vectors, cells, and compositions comprising such molecules, are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such NCSM molecules, in the context of the present invention, to a patient are available, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route. Preferred routes are readily ascertained by those of skill in the art.

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Compositions comprising cells expressing at least one full length form of a NCSM polypeptide or a fragment thereof (ECD) are also a feature of the invention. Such cells may also express one or more antigens specific for the intended application (e.g., cancer antigen). Such cells are readily prepared as described herein by transfection with

5   DNA plasmid vector encoding at least one of the NCSM polypeptide and/or antigen. Separate vectors each encoding a NCSM polypeptide and antigen may be used to transfect the cells, or a bicistronic vector encoding both the NCSM polypeptide and antigen can be used. Compositions of such cells may be pharmaceutically compositions further comprising a pharmaceutically acceptable carrier or excipient.

10   Pharmaceutical compositions of the invention can, but need not, include a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention. A variety of aqueous

15   carriers can be used, e.g., buffered saline, such as PBS, and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like,

20   for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of gene therapy or genetic vaccine vector in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

25   Compositions comprising NCSM polypeptides and polynucleotides, and vectors, cells, and other formulations comprising these and other components of the invention, can be administered by a number of routes including, but not limited to oral, intranasal, intravenous, intraperitoneal, intramuscular, transdermal, subcutaneous, intradermal, topical, systemic, mucosal, inhalation, parenteral, sublingual, vaginal, or rectal

30   means. Polypeptide and nucleic acid compositions can also be administered via liposomes. Such administration routes and appropriate formulations are generally known to those of skill in the art.

The NCSM polypeptide or polynucleotide or fragment thereof, or vector comprising a NCSM nucleic acid, alone or in combination with other suitable components, can also be made into aerosol formulations (e.g., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable

5   propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c)

10   suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, tragacanth, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives,

15   flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art. It is recognized that the gene

20   therapy vectors and genetic vaccines, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the vector with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the vector in an appropriately resistant carrier such as a liposome. Means of protecting vectors from digestion are well known in the art. The pharmaceutical compositions can be

25   encapsulated, e.g., in liposomes, or in a formulation that provides for slow release of the active ingredient.

The packaged nucleic acids, alone or in combination with other suitable components, can be made into aerosol formulations (e.g., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable

30   propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the packaged nucleic acid with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In

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addition, it is also possible to use gelatin rectal capsules which consist of a combination of the packaged nucleic acid with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, subdermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, mucosally, topically, intraperitoneally, intravesically or intrathecally. The formulations of packaged nucleic acids or polypeptides of the invention can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

Parenteral administration and intravenous administration are preferred methods of administration. In particular, any routes of administration already in use for existing co-stimulatory therapeutics and prophylactic treatment protocols, including those currently employed with e.g., mammalian B7-1 polynucleotides and polypeptides, such as hB7-1, along with pharmaceutical compositions and formulations in current use, are preferred routes of administration and formulation for the NCSM polynucleotides or polypeptides (and fragments thereof).

Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by the packaged nucleic acid can also be administered intravenously or parenterally.

Cells transduced with the NCSM nucleic acids as described herein in the context of ex vivo or in vivo therapy can also be administered intravenously or parenterally. It will be appreciated that the delivery of cells to patients is routine, e.g., delivery of cells to the blood via intravenous, intramuscular, or intraperitoneal administration or other common route.

The dose administered to a patient, in the context of the present invention is sufficient to effect a beneficial effect, such as an altered immune response or other therapeutic and/or prophylactic response in the patient over time, or to, e.g., inhibit infection by a pathogen, depending on the application. The dose will be determined by the efficacy

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of the particular nucleic acid, polypeptide, vector, composition or formulation, transduced cell, cell type, and/or the activity of the NCSM polypeptide and/or polynucleotide included therein or employed, and the condition of the patient, as well as the body weight, surface area, or vascular surface area, of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of any such particular polypeptide, nucleic acid, vector, formulation, composition, transduced cell, cell type, or the like in a particular patient. Dosages to be used for therapeutic or prophylactic treatment of a particular disease or disorder can be determined by one of skill by comparison to those dosages used for existing therapeutic or prophylactic treatment protocols for the same disease or disorder.

Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by the packaged nucleic acid can also be administered intravenously or parenterally.

In determining the effective amount of the vector, cell type, composition, or formulation to be administered to a subject for the treatment or prophylaxis of the medical condition or disease state (e.g., cancers or viral diseases), a physician evaluates the subject for, e.g., circulating plasma levels, vector/cell/formulation/NCSM molecule toxicities, progression of the disease or condition, and the production of anti-vector/ NCSM polypeptide antibodies, and depending on the subject other factors that would be known to one of skill in the art.

In one aspect, for example, in determining the effective amount of the vector to be administered in the treatment or prophylaxis of an infection or other condition, wherein the vector comprises any NCSM nucleic acid sequence described herein or encodes any NCSM polypeptide described herein, the physician evaluates vector toxicities, progression of the disease, and the production of anti-vector antibodies, if any. In one aspect, the dose equivalent of a naked nucleic acid from a vector for a typical 70 kilogram patient can range from about 10 ng to about 1 g, about 100 ng to about 100 mg, about 1 μg to about 10 mg, about 10 μg to about 1 mg, or from about 30-300 μg. Doses of vectors used to deliver the nucleic acid are calculated to yield an equivalent amount of therapeutic nucleic acid. Administration can be accomplished via single or divided doses.

In another aspect, the dose administered, e.g., to a 70 kilogram patient can be in the range equivalent to any dosages of currently-used co-stimulatory or WT B7-1 therapeutic or prophylactic proteins (such a hB7-1) or the like, and doses of vectors or cells

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which produce NCSM sequences optionally are calculated to yield an equivalent amount of NCSM nucleic acid or expressed polypeptide or protein. The vectors of this invention comprising at least one nucleotide sequence encoding at least one NCSM (and, if desired, further comprising a nucleotide sequence encoding antigen or other co-stimulatory molecule

5   either on the same vector) can be used to prophylactically or therapeutically treat or supplement such treatment of a variety of cancers, including e.g., colorectal cancer, breast cancer, pancreatic cancer, lung cancer, prostate cancer, naso-pharyngeal cancer, cancer, brain cancer, leukemia, melanoma, head- and neck cancer, stomach cancer, cervical cancer, ovarian cancer, lymphomas, colon cancer, colorectal, and virally-mediated conditions by

10   any known conventional therapy, including cytotoxic agents, nucleotide analogues (e.g., when used for treatment of HIV infection), biologic response modifiers, and the like.

In therapeutic applications, compositions are administered to a patient suffering from a disease (*e.g.,* an infectious disease, cancer, or autoimmune disorder) in an amount sufficient to cure or at least partially arrest or ameliorate the disease or at least one

15   of its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health. Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity

20   of protein to effectively treat the patient.

In prophylactic applications, compositions are administered to a human or other mammal to induce an immune or other prophylactic response that can help protect against the establishment of an infectious disease, cancer, autoimmune disorder, or other condition.

25   In some applications, an amount of NCSM polypeptide that is administered to a subject for a particular therapeutic or prophylactic treatment protocol or vaccination ranges from about 1 to about 50 mg/kg weight of the subject. Such amount of polypeptide can be administered 1 time/week or up to 3 times/week, as desired. Such NCSM polypeptide can be administered as a soluble molecule comprising, e.g., an NCSM-ECD, or

30   NCSM-trunECD-Ig or NCSM-ECD-Ig fusion protein. Alternatively, such NCSM polypeptide can be administered in the form of a NCSM-polypeptide-encoding polynucleotide, which is operably linked to a promoter, such that the polynucleotide expresses in the subject such a NCSM polypeptide of from about 1 to about 50 mg/kg

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weight of the subject (e.g., on the surface of targeted cells) or as an expressed soluble NCSM polypeptide. The NCSM polypeptide (or nucleic acid encoding the polypeptide) can be administered to a population of cells of a subject in vivo, or to a population of cells of the subject ex vivo as described herein. Compositions comprising soluble NCSM polypeptides

5   in such range amounts or comprising nucleic acids or expression vectors that can express such amounts in the subject are also contemplated.

In cancer immunotherapy or prophylactic applications (e.g., multiple myeloma, breast cancer, lymphoma, and the like), it is advantageous to administer at least one NCSM molecules (including, e.g., CD28BPs and CTLA-4BPs) and at least one cancer

10   antigen with at least one other molecule of interest, such as, e.g., a cytokine (IL-12, IL-15, IL-2, or variant thereof, etc.) and/or colony stimulating factor (e.g., GM-CSF); such combination can serve to enhance a desired response, e.g., to enhance lymphocyte proliferation and/or gamma-interferon release. Included are recombinant, variant and mutant forms of IL-12, including recombinant IL-12p-40 and IL-12p35 polypeptides and

15   nucleic acids described in PCT App. No. US00/32664 (Publ. No. WO 01/40257), which is incorporated herein by reference in its entirety for all purposes. In one format, a bicistronic vector comprising nucleotide sequences encoding an NCSM polypeptide, cancer antigen, and polypeptide(s) of interest is administered to the subject (e.g., by intramuscular or intradermal injection). In another format, a vector comprising a nucleotide sequence

20   encoding the molecule of interest can be administered separately to the patient, at the same time or following administration of the one or more vectors comprising sequences encoding the antigen and NCSM polypeptide. Typically, a dose of at least about 1 mg nucleic acid (e.g., DNA) of GM-CSF and/or IL-2, IL-12 or other cytokine is administered at the time of immunization with the antigen and NCSM nucleic acids. Alternatively, the additional

25   molecule of interest (GM-CSF, IL-12, IL-2, or other cytokine) is administered to the subject as a polypeptide (e.g., by i.m. or i.d. injection). The initial dose of this polypeptide is administered at about the same time as the vector encoding the NCSM polypeptide and antigen, and typically comprises at least about 75 ug. Subsequent additional "boost" doses of at least about 75 ug are usually delivered once/day for at least four days following the

30   initial immunization. In another format, one or more vectors encoding either or both an NCSM polypeptide and molecule of interest (cytokine, GM-CSF) are administered (via, e.g., i.d. or i.m. injection) in vivo into the tumor of a subject where the tumor is inoperable, or into tumor cells removed from a patient (ex vivo administration). Additional vector

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formats can also be used (adenoviral, retroviral, bicistronic, tricistronic).The toxicity and therapeutic efficacy of the vectors that include recombinant molecules provided by the invention are determined using standard pharmaceutical procedures in cell cultures or experimental animals. One can determine the LD_{50} (the dose lethal to 50% of the

5   population) and the ED_{50} (the dose therapeutically effective in 50% of the population) using procedures presented herein and those otherwise known to those of skill in the art. Nucleic acids, polypeptides, proteins, fusion proteins, transduced cells and other formulations of the present invention can be administered at a rate determined, e.g., by the LD_{50} of the formulation, and the side-effects thereof at various concentrations, as applied to the mass

10   and overall health of the patient. Again, administration can be accomplished via single or divided doses.

A typical pharmaceutical composition for intravenous administration is about 0.1 to 10 mg per patient per day. Dosages from 0.1 up to about 100 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the

15   blood stream, such as into a body cavity or into a lumen of an organ. Substantially higher dosages are possible in topical administration. For recombinant promoters of the invention that express the linked transgene at high levels, it may be possible to achieve the desired effect using lower doses, e.g., on the order of about 1 μg or 10 μg per patient per day. Actual methods for preparing parenterally administrable compositions will be known or

20   apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980).

For introduction of recombinant NCSM nucleic acid transduced cells into a patient, an illustrative, but not limiting example includes taking blood samples, obtained

25   prior to infusion, and saved for analysis. Between, e.g., 1×10^6 and 1×10^{12} transduced cells are infused intravenously over, e.g., 60-200 minutes. Vital signs and oxygen saturation by pulse oximetry are closely monitored. Blood samples are obtained, e.g., 5 minutes and, e.g., 1 hour following infusion and saved for subsequent analysis. Leukopheresis, transduction and reinfusion are optionally repeated every, e.g., 2 to 3

30   months for a total of, e.g., 4 to 6 treatments in a one year period. After the first treatment, infusions can be performed, e.g., on a outpatient basis at the discretion of the clinician. If the reinfusion is given as an outpatient, the participant is monitored for, e.g., at least 4, and preferably, e.g., 8 hours following the therapy. Transduced cells are prepared for reinfusion

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according to established methods. *See,* Abrahamsen et al. (1991) <u>J Clin Apheresis</u> 6:48-53;
Carter et al. (1988) <u>J Clin Arpheresis</u> 4:113-117; Aebersold et al. (1988), <u>J Immunol</u>
<u>Methods</u> 112:1-7; Muul et al. (1987) <u>J Immunol Methods</u> 101:171-181 and Carter et al.
(1987) <u>Transfusion</u> 27:362-365. After a period of, e.g., about 2-4 weeks in culture, the cells

5   should number between, e.g., 1×10^6 and 1×10^{12}. In this regard, the growth characteristics
of cells vary from patient to patient and from cell type to cell type. About, e.g., 72 hours
prior to reinfusion of the transduced cells, an aliquot is taken for analysis of phenotype, and
percentage of cells expressing the therapeutic agent.

If a patient undergoing infusion of a vector or transduced cell or protein

10   formulation develops, e.g., fevers, chills, or muscle aches, he/she receives the appropriate
dose of, e.g., aspirin, ibuprofen, acetaminophen or other pain/fever controlling drug.
Patients who experience reactions to the infusion such as fever, muscle aches, and chills are
premedicated, e.g., 30 minutes prior to the future infusions with, e.g., either aspirin,
acetaminophen, or, e.g., diphenhydramine, etc. Meperidine is used for more severe chills

15   and muscle aches that do not quickly respond to antipyretics and antihistamines. Cell
infusion is, e.g., slowed or discontinued depending upon the severity of the reaction.

The NCSM polypeptides, NCSM nucleic acids, and cells, vectors, transgenic
animals, and compositions that include the NCSM molecules of the invention can be
packaged in packs, dispenser devices, and kits for administration to a subject, such as a

20   mammal. For example, packs or dispenser devices that contain one or more unit dosage
forms are provided. Typically, instructions for administration of the compounds will be
provided with the packaging, along with a suitable indication on the label that the
compound is suitable for treatment of an indicated condition. For example, the label may
state that the active compound within the packaging is useful for treating a particular

25   infectious disease, autoimmune disorder, tumor, or for preventing or treating other diseases
or conditions that are mediated by, or potentially susceptible to, a subject's or mammalian
immune response.

Any NCSM nucleic acid, polypeptide, protein, fusion protein, or vector or
comprising any such NCSM molecule described herein, and any composition comprising at

30   least one NCSM nucleic acid, polypeptide, protein, fusion protein, or vector or cell
comprising at least one such NCSM molecule, can be used in any of the methods and
applications described herein. In one aspect, the invention provides for the use of any
NCSM polypeptide or nucleic acid (or vector or cell comprising a NCSM nucleic acid) or

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composition thereof as a medicament, or as a vaccine, for the treatment of one of the diseases described herein or for preventing one of the diseases described herein, or the like. In another aspect, the invention provides for the use of any NCSM polypeptide or nucleic acid (or vector or cell comprising a NCSM nucleic acid) or composition thereof for the

5   manufacture of a medicament, prophylactic, therapeutic, drug, or vaccine, including for any therapeutic or prophylactic application relating to treatment of a disease or disorder as described herein.

In one aspect, the invention provides methods for modulating or altering an immune response T-cell response specific to an antigen in a subject. Some such methods

10   comprise administering to the subject at least one polynucleotide sequence comprising a NCSM polynucleotide described here (e.g., SEQ ID NOS:1-47, 95-173, and 253-262, 274-277) or at least one polynucleotide encoding a polypeptide comprising any of SEQ ID NOS:48-94, 174-252, 263-272, 279-293 or fragment thereof, and a polynucleotide sequence encoding the antigen or antigenic fragment thereof. Each of the at least one polynucleotide

15   sequences is expressed in the subject in an amount effective to modulate or alter an immune response or a T cell response. In some such methods, the polypeptide or fragment thereof interacts with or binds a T cell surface receptor. In some such methods, T-cell response is enhanced as measured by assays described herein, and in some such methods, the enhanced T cell response is sufficient to eliminate cells bearing the antigen or antigenic fragment

20   thereof. In other methods, the T-cell response is suppressed or inhibited as measured by assays described herein.

In some such methods, the antigen or antigenic fragment thereof is an antigen or antigenic fragment thereof of an infectious agent or a cancer. The encoded polypeptide may comprising any NCSM polypeptide of fragment thereof described herein,

25   such as SEQ ID NO:66 or SEQ ID NO:86, or the extracellular domain amino acid sequence of any NCSM polypeptide described herein, or fusion protein thereof.

The at least one polynucleotide sequence encoding a NCSM polypeptide or fragment thereof may be operably linked to a promoter in a vector, such as an expression vector or DNA plasmid. In one aspect, the at least one polynucleotide sequence encoding

30   the antigen or antigenic fragment thereof may be included in the same vector and operably linked to a second promoter in the same vector (e.g., bicistronic vector). Alternatively, the polynucleotide sequences encoding the NCSM polypeptide and the antigen or antigenic fragment are present in separate vectors and administered separately, e.g., either

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simultaneously or consecutively. The antigen or antigenic fragment thereof may thus be operably linked to a promoter in the second vector.

In another aspect, the invention provides vectors comprising at least one NCSM polynucleotide sequence described herein (e.g., SEQ ID NOS1-47, 95-173, and 253-262) or a polynucleotide sequence encoding a polypeptide comprising any of SEQ ID NOS:48-94, 174-252, 263-272 and 283-293 or fragment thereof, and a polynucleotide sequence encoding the antigen or antigenic fragment thereof, wherein the NCSM polypeptide or fragment thereof interacts with or binds to a T cell receptor when expressed in a subject, and wherein each of the at least one polynucleotide sequences is operably linked to a promoter for expression in the subject and is present in an amount sufficient that when expressed is effective to modulate or alter a T cell response.

In some such methods, the at least one polynucleotide sequence encoding a polypeptide comprises a polynucleotide sequence of any of SEQ ID NOS:1-47, 95-173, and 253-262. Each of the at least one polynucleotide sequences may be expressed in the subject in an amount effective to enhance a T cell response such that cells expressing the antigen or antigenic fragment thereof are eliminated. In some methods, each of the at least one polynucleotide sequences is expressed in the subject in an amount effective to inhibit a T cell response.

In another aspect, the invention provides vectors comprising at least one NCSM polynucleotide sequence described herein or at least one polynucleotide sequence encoding a polypeptide comprising any of SEQ ID NOS:48-94, 174-252, 263-272 and 283-293 or fragment thereof, wherein the polypeptide or fragment thereof interacts with or binds to a T cell receptor when expressed in a subject, wherein the at least one polynucleotide sequence is operably linked to a promoter for expression in the subject and is present in an amount sufficient that when expressed is effective to modulate or alter a T cell response.

In yet another aspect, the invention provides methods of modulating or altering an immune response in a subject, the method comprising introducing into cells of a tumor of the subject at least one polynucleotide sequence encoding a polypeptide comprising any of SEQ ID NOS:48-94, 174-252, 263-272 and 283-293 or fragment thereof, wherein the polypeptide or fragment thereof interacts with or binds to a T cell receptor when expressed in a subject, and wherein the at least one polynucleotide sequence is operably linked to a promoter for expression in the subject and present in an amount sufficient that when expressed is effective to modulate or alter a T cell response.

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The invention includes therapeutic methods for activating or enhancing a T-cell response in a subject, wherein the subject may have a tumor or from whom a tumor was surgically removed. Such methods comprise administering to the subject a composition that comprises a polynucleotide sequence encodes a NCSM polypeptide and an excipient,

5   wherein the NCSM polypeptide is expressed by tumor cells or tumor-related cells of the subject, and the T-cell response is activated or enhanced against the tumor. For some such methods, the polynucleotide sequence encodes a soluble NCSM polypeptide. The composition may comprise a vector comprising the polynucleotide sequence that encodes a NCSM polypeptide. Further, a therapeutically effective amount of the composition

10   sufficient to enhance a T-cell response against the tumor may be administered. A pharmaceutical composition comprising an expression vector comprising a polynucleotide sequence that encodes a NCSM polypeptide and a pharmaceutically acceptable excipient are also provided.

The invention also includes therapeutic methods for activating or enhancing

15   a T-cell response in a subject who has a tumor or from whom a tumor was removed surgically, the method comprising administering to the subject a composition that comprises a soluble NCSM polypeptide and an excipient, wherein the T-cell response is activated or enhanced against the tumor. Also included are methods for activating or enhancing a T-cell response in such a subject, the methods comprising administering to the subject a sufficient

20   amount of a composition comprising an excipient and a population of cells expressing a NCSM polypeptide and an antigen, such that the T-cell response is thereby activated or enhanced against the tumor. Also contemplated are methods for activating or enhancing a T-cell response in such a subject, the method comprising administering to the subject a sufficient amount of a composition comprising an excipient and a population of cells

25   expressing a NCSM polypeptide, such that the T-cell response is thereby activated or enhanced against the tumor.

USES AND APPLICATIONS

The evolved novel NCSM molecules of the invention, in all formats described herein, including, but not limited to, e.g., NCSM nucleic acids, NCSM

30   polypeptides and proteins, and vectors, cells, compositions including such NCSM molecules, are useful in a broad range of clinical, therapeutic, and prophylactic applications. Optionally, the polypeptides alone or fragments thereof are used to enhance the immune

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system (e.g., NCSM polypeptides or soluble NCSM polypeptides (e.g., ECD), NCSM fusion proteins (e.g., comprising an NCSM-ECD fused to an Ig)). For example, these molecules are useful in enhancing tumor immunity and as polypeptide or protein adjuvants and DNA vaccine adjuvants in combination with, e.g., antigens for specific diseases. For

5   example, CD28BP polypeptides and nucleic acids encoding CD28BP polypeptides are useful are adjuvants to enhance or boost an immune response in a subject, including to augment or enhance a response to a particular compound that is delivered to the subject simultaneously or before or after the CD28BP adjuvant. They are also useful in the treatment of a variety of medical conditions, including, e.g., chronic infectious diseases,

10   allergies, autoimmune diseases, and in organ transplantation and the reversal of septic shock. Moreover, transgenic animals, such as pigs, mice, etc., expressing CD28BP and/or CTLA-4BP can be generated using methods known to those skilled in the art. Proteins, tissues or organs from such animals can be used to modulate T cell responses in patients undergoing tissue or organ transplantation.

15   Furthermore, NCSM molecules, such as the CD28BP and CTLA-4BP molecules described herein, are useful as components in multi-component vaccines, which optionally comprise, e.g., a single vector with multiple components or multiple vectors encoding different vector components or a multi-component protein-based vaccines in which a CD28BP or CTLA-4BP protein is delivered with other proteins, such as a protein

20   vaccine. The CD28BP or CTLA-4BP protein can be delivered simultaneously with the other protein(s) if desired, or delivered at a different time, and can also be administered to a subject following delivery of a protein vaccine or DNA vaccine to boost the immune response to the protein vaccine or DNA vaccine. A multi-component vaccine optionally comprises, e.g., a vector, such as a DNA plasmid vector, that comprises, for example, in

25   addition to nucleotide sequences encoding one or more CD28BP and/or CTLA-4BP polypeptides, one or more nucleotide sequences encoding at least of the following components: at least one antigen(s), cytokine(s), adjuvant(s), promoter (e.g., wild-type CMV promoter (such as human CMV promoter with or without an intron A sequence; or a recombinant, or chimeric CMV promoter with or without a recombinant or WT intron A

30   sequence), and/or other co-stimulatory molecule(s) (each of which may have been optimized by recursive sequence recombination and selection/screening procedures, random mutagenesis, or other known mutagenesis procedures), and combinations of such various components. Such multi-component vector expresses two or more such components and

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includes appropriate expression elements for such expression (see, e.g., an exemplary multi-component vector described in Example V). Such an arrangement permits co-delivery of various components, including recursively recombined components, for a particular treatment regimen or therapeutic or prophylactic application. Such vectors are designed according to the specific treatment regimen or therapeutic or prophylactic application desired. One or more such single-component or multi-component vectors as described above may be used simultaneously or in sequential administration in a therapeutic or prophylactic treatment method of the invention.

Also, an immune response is optionally modified or enhanced by, e.g., administering one or more nucleic acids encoding one or more novel CD28BPs (or fragments thereof, including, e.g., soluble CD28BPs or fusion proteins thereof) or CTLA-4BPs (or fragments thereof, including, e.g., soluble CTLA-4BPs or fusion proteins thereof) with an antigen. Alternatively, an antigen response is optionally enhanced or modified by administration of one or more CD28BPs (or fragments thereof, including, e.g., soluble CD28BPs) or CTLA4-BPs (or fragments thereof, including, e.g., soluble CTLA-4BPs) with an antigen.

CD28BPs and CTLA-4BPs, and B7-1 and B7-2 polypeptide variants described herein (and nucleic acids encoding any of these polypeptides or fragments thereof), are useful in modulating the immune response in vivo in a variety of animals, (e.g., mammals, (including humans)) and in vitro. These molecules are particularly useful in therapeutic and/or prophylactic applications when modulation of T cell responses is desired. Examples of useful applications for CD28BP and/or CTLA4BP (or fragments thereof of each, or soluble and/or fusion protein versions of each) include conditions or diseases that may benefit from enhanced T cell responses or where enhanced T cell responses are desired. They are also useful, for example, in treating diseases where inhibition of T cell proliferation/activation is desired. Examples of medical conditions and/or diseases where enhanced T cell response is desired (e.g., by use of CD28BPs) (or fragments thereof, soluble and/or fusion protein versions) include, for example, cancer, chronic infectious diseases, and vaccinations. Cancers include, but are not limited to, e.g., colorectal cancer, breast cancer, pancreatic cancer, lung cancer, prostate cancer, naso-pharyngeal cancer, cancer, brain cancer, leukemia, melanoma, head- and neck cancer, stomach cancer, cervical cancer, ovarian cancer, and lymphomas.

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CD28BPs, CTLA-4BPs, and B7-1 and B7-2 polypeptide variants described herein (and nucleic acids encoding any of these), are useful in a variety of therapeutic and prophylactic treatment of diseases and conditions, including, e.g., allergy/asthma, neurological, organ transplantation (e.g., graft versus host disease, and autoimmune

5   diseases), malignant diseases, chronic infectious diseases, including, but not limited to, e.g., viral infectious diseases, such as those associated with, but not limited to, e.g., hepatitis B virus (HBV), herpes simplex virus (HSV), hepatitis C virus (HCV), HIV, human papilloma virus (HPV), and the like, and bacterial infectious diseases, such as, but not limited to, e.g., Lyme disease, tuberculosis, and chlamydia infections, and the like.

10   Furthermore, CD28BPs, CTLA-4BPs, and B7-1 and B7-2 polypeptide variants described herein (and nucleic acids encoding these) are useful in methods for modulating production of specific cytokines, including those discussed in the Examples below. These molecules are particularly useful in therapeutic and/or prophylactic applications in which an adjustment, alteration of a cytokine level, or production or

15   stimulation of a specific cytokine production is desired.

CD28BP polypeptides of the invention, or fragments thereof or soluble and/or fusion proteins thereof, modulate T cell proliferation or activation and augment the immune response. In one embodiment, such a CD28BP polypeptide can be delivered in a treatment protocol as a component of a DNA vaccine vector, as a full-length polypeptide, as

20   a soluble polypeptide subsequence of the full-length CD28BP polypeptide (e.g., ECD) used, if desired, as a polypeptide or protein vaccine or "boosting" polypeptide, or as a soluble fusion protein comprising a full-length CD28BP polypeptide or subsequence thereof, such as a soluble polypeptide subsequence (e.g., ECD); in such formats, the CD28BP polypeptide may act as an agonist. In another embodiment, such as a genetic vaccine, in

25   combination with a nucleic acid sequence encoding a specific antigen, a nucleic acid sequence encoding a CD28BP polypeptide augments the antigen specific T cell response for infectious disease or cancer antigens.

The CTLA4BPs, or fragments thereof or soluble and/or fusion proteins thereof, of the invention can modulate T cell proliferation and/or activation and inhibit the

30   immune response in autoimmune diseases or, as soluble molecules, act as antagonists. Such a CTLA-4BP polypeptide can be delivered in a treatment protocol as a component of a DNA vaccine vector, as a full-length polypeptide, as a soluble polypeptide subsequence of the full-length CTLA-4BP polypeptide (e.g., ECD) used, if desired, as a polypeptide or

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protein vaccine or "boosting" polypeptide, or as a soluble fusion protein comprising a full-length CTLA-4BP polypeptide or subsequence thereof, such as a soluble polypeptide subsequence (e.g., ECD); in such formats, the CTLA-4BP polypeptide may serve as an agonist.

5   As discussed above, genetic vaccine comprising a vector comprising a nucleic acid sequence encoding a CTLA4-BP polypeptide and at least one nucleic acid sequence encoding at least one additional polypeptide of interest is also a feature of the invention. For example, in a DNA vaccine, in combination with a specific allergen, the CTLA4BPs (or fragments thereof, or soluble and/or fusion proteins thereof) may inhibit the 10   allergen specific T cell response in allergy. Similarly, in combination with a specific auto-antigen, such as myelin basic protein, the CTLA-4BPs (or fragments thereof, or soluble and/or fusion proteins thereof) may inhibit the auto-antigen-specific T cell response in autoimmunity, such as in multiple sclerosis.

        Other clinical applications in which inducing tolerance is important and thus 15   in which CTLA-4BPs are of use include autoimmunity (e.g., multiple sclerosis, rheumatoid arthritis, psoriasis), severe allergy and asthma, organ transplantation, generation of transgenic tissues to enable xenotransplants, graft versus host disease, and with components of gene therapy vectors to prolong survival of cells expressing foreign proteins.

        Examples of medical conditions and/or diseases where down-regulation, or 20   other altered type of T cell function by delivery of a CTLA-4BP of the invention either as a DNA expression vector comprising a nucleic acid sequence encoding a CTLA-4BP polypeptide or as a soluble described herein), or fragments thereof or soluble and/or fusion proteins thereof, may be of benefit include allergy, undesired immune response, autoimmune diseases, septic shock, and organ transplantation.

25   Examples of useful pathogen antigens, cancer antigens, allergens and auto-antigens for use in methods of the invention and/or in combination with NCSM polypeptides have been provided in the following commonly assigned patent applications: Punnonen et al. (1999) WO 99/41369; Punnonen et al. (1999) WO 99/41383; Punnonen et al. (1999) WO 99/41368; and Punnonen et al. (1999) WO 99/41402), each of which is 30   incorporated herein by reference in its entirety for all purposes. Several other useful antigens have been described in the literature or can be discovered using genomics approaches. Since typical tumor antigens are self proteins and thus host tolerant, it is optionally necessary to generate "non-self" tumor antigens that induce cross-reactivity

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against self tumor antigens also. This is optionally accomplished through, e.g., recursive sequence recombination of existing tumor antigens from diverse species to produce chimeric tumor antigens. Such chimeric antigens are then screened for ones which activate antigen-specific T cells which also recognize the wild-type tumor antigen. Optional

5   screenings test whether chimeric antigens activate patient T cells (e.g., T cell lines specific for wild-type antigens generated and activation induced by APCs expressing recursively recombined antigens analyzed) and whether the chimeric antigen induces T cells that recognize wild-type antigen (e.g., T cell lines specific for recursively recombined antigens generated and activation induced by APCs expressing WT antigen analyzed).

10   A NCSM polypeptide of the invention is also useful in therapeutic or prophylactic treatment methods for treating or preventing any of the above-mentioned diseases and disorders when administered to a subject as a polypeptide (e.g., administer at least one full-length or soluble NCSM or fragment thereof) or cell-based vaccine (e.g., cell expressing or secreting at least one NCSM polypeptide) or a gene-based therapeutic

15   polypeptide (i.e., polypeptide product expressed by a NCSM gene), wherein such NSMC polypeptides are delivered alone or co-administered simultaneously or subsequently with one or more of an antigen, another co-stimulatory molecule, or adjuvant. A NCSM molecule is also useful for treating or preventing any of the above-mentioned diseases and disorders when administered to a subject as a genetic vaccine (e.g., DNA vaccine) in which

20   at least one NCSM polynucleotide is administered alone or in a plasmid vector or gene therapy format (i.e., a vector encoding at least one NCSM polypeptide). Or, if desired, at least one NCSM polynucleotide is co-administered with a second DNA vector encoding at least one of an antigen, co-stimulatory molecule, and/or adjuvant. Alternatively, if desired, a vector comprising at least one NCSM-encoding polynucleotide sequence and at least one

25   of an antigen, co-stimulatory molecule, and/or adjuvant can be prepared and administered to a subject in a treatment protocol; in this instance, the at least one NCSM-encoding polynucleotide is co-expressed with at least one antigen, co-stimulatory molecule, and/or adjuvant.

In another aspect, a soluble NCSM polypeptide may be used in methods for

30   treating or preventing any of the above-mentioned diseases or disorders when administered to a subject alone or in conjunction simultaneously or subsequently with one or more of an antigen, another co-stimulatory, and/or adjuvant. The soluble NCSM may comprise a NCSM-ECD or subsequence thereof or may be formulated as a soluble fusion protein.

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Further, a NCSM polynucleotide that encodes a NSCM or any soluble form of a NCSM as described herein is useful in therapeutic or prophylactic treatment methods for treating or preventing any of the above-mentioned diseases or disorders when administered to a subject alone or in conjunction (simultaneously or subsequently) with one or more nucleotide

5   sequences encoding one or more of an antigen, another co-stimulatory, and/or adjuvant. If desired, the NCSM polynucleotide sequence and one or more polynucleotide sequences encoding one or more of an antigen, another co-stimulatory, and/or adjuvant may be incorporated into one vector for delivery to the subject and co-expression of the resulting NCSM polypeptide, antigen, another co-stimulatory, and/or adjuvant.

10   As noted above, a variety of antigens can be delivered simultaneously with or following delivery of a NCSM molecule, where the NCSM molecule is administered to the subject in either polypeptide or nucleic acid format. The antigen may be delivered as a polypeptide or polynucleotide (via a vector). Antigens may be WT antigens or recombinant or chimeric antigens, including, e.g., shuffled or mutated antigens.

15   Examples of cancer antigens that be used with NCSM molecules and in methods of the invention described herein include, e.g., EpCam/KSA, bullous pemphigoid antigen 2, prostate mucin antigen (PMA) (Beckett and Wright (1995) Int. J. Cancer 62:703-710), tumor associated Thomsen-Friedenreich antigen (Dahlenborg et al. (1997) Int. J. Cancer 70:63-71), prostate-specific antigen (PSA) (Dannull and Belldegrun (1997) Br. J.

20   Urol. 1:97-103), luminal epithelial antigen (LEA.135) of breast carcinoma and bladder transitional cell carcinoma (TCC) (Jones et al. (1997) Anticancer Res. 17:685-687), cancer-associated serum antigen (CASA) and cancer antigen 125 (CA 125) (Kierkegaard et al. (1995) Gynecol. Oncol. 59:251-254), the epithelial glycoprotein 40 (EGP40) (Kievit et al. (1997) Intl. J. Cancer 71:237-245), squamous cell carcinoma antigen (SCC) (Lozza et al.

25   (1997) Anticancer Res. 17: 525-529), cathepsin E (Mota et al. (1997) Am. J. Pathol. 150:1223-1229), tyrosinase in melanoma (Fishman et al. (1997) Cancer 79: 1461-1464), cell nuclear antigen (PCNA) of cerebral cavernomas (Notelet et al. (1997) Surg. Neurol. 47: 364-370), DF3/MUC1 breast cancer antigen (Apostolopoulos et al. (1996) Immunol. Cell. Biol. 74: 457-464; Pandey et al. (1995) Cancer Res. 55: 4000-4003), carcinoembryonic

30   antigen (Paone et al. (1996) J. Cancer Res. Clin. Oncol. 122:499-503; Schlom et al. (1996) Breast Cancer Res. Treat. 38:27-39), tumor-associated antigen CA 19-9 (Tolliver and O'Brien (1997) South Med. J. 90:89-90; Tsuruta et al. (1997) Urol. Intl. 58:20-24), human melanoma antigens MART-1/Melan-A27-35 and gp100 (Kawakami and Rosenberg (1997)

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Intl. Rev. Immunol. 14:173-192; Zajac et al. (1997) Intl. J. Cancer 71:491-496), the T and Tn pancarcinoma (CA) glycopeptide epitopes (Springer (1995) Crit. Rev. Oncog. 6:57-85), a 35 kD tumor-associated autoantigen in papillary thyroid carcinoma (Lucas et al. (1996) Anticancer Res. 16:2493-2496), KH-1 adenocarcinoma antigen (Deshpande and

5   Danishefsky (1997) Nature 387:164-166), the A60 mycobacterial antigen (Maes et al. (1996) J. Cancer Res. Clin. Oncol. 122:296-300), heat shock proteins (HSPs) (Blachere and Srivastava (1995) Semin. Cancer Biol. 6:349-355), and MAGE, tyrosinase, melan-A and gp75 and mutant oncogene products (e.g., p53, ras, CDk4, and HER-2/neu (Bueler and Mulligan (1996) Mol. Med. 2:545-555; Lewis and Houghton (1995) Semin. Cancer Biol. 6:

10   321-327; Theobald et al. (1995) Proc. Nat'l. Acad. Sci. USA 92: 11993-11997), prostate specific membrane antigen (PSMA) Bangma CH et al. (2000) Microsc Res Tech 51:430-5, TAG-72, McGuinness RP et al. Hum Gene Ther (1999) 10:165-73, and variants, derivatives, and mutated, and recombinant forms (e.g., shuffled forms) thereof of these antigens.

15   To generate, e.g., vaccines with evolved NCSM molecules, pre-clinical studies can first be done in, e.g., mice. Mice can be used for study of, e.g., CTLA-4BPs because, e.g., effects of the protein are more difficult to study in vitro, work in monkeys is less cost effective than work with mice, mouse models of autoimmune diseases have been established giving excellent means to study induction and breaking tolerance, and the same

20   mouse models can be used for biological characterization of CD28BPs as well. Pre-clinical mice studies can allow optimization of, e.g., vectors for specific targets of interest, pharmacokinetics, drug half life, adjuvant stability and in vivo efficacy of such things as DNA vaccines and soluble protein administration. Mouse studies optionally can be followed by pre-clinical studies in non-human primates. Non-human primate trials can,

25   e.g., optimize efficacy in boosting the innate immune system as well as optimize efficacy of use of NCSM molecules as vaccine adjuvants. Non-human primate trials optionally can serve to, e.g., optimize protective immunity and thereby help identify the best vaccine for human clinical trials.

             As an illustrative, but not limiting example, either or both types of NCSM

30   (i.e., CD28BPs and CTLA-4BPs) or fragments thereof can be used in a boosting method or format to modify an immune response. This method typically comprises, e.g., initially administering a DNA vaccine (a "prime boost") to a subject, followed by, e.g., a second

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administration with, e.g., one or more of the NCSM polypeptide molecules either in polypeptide format or in nucleic acid format.

Furthermore, the invention also provides for gene therapy vectors comprising at least one nucleotide sequence encoding at least one CTLA-4BP or fragment, variant or homologue thereof. In one aspect, a gene therapy vector (e.g., adenovirus (AV), adeno-associated virus (AAV), retrovirus, poxvirus, or lentivirus vectors) comprising at least one nucleic acid sequence encoding at least one CTLA-4BP polypeptide or fragment thereof) is used to reduce recognition of the transduced cells by specific T cells. The incorporation of the CTLA-4BP-encoding nucleic acid sequence helps to prolong survival of the gene therapy vector. In gene therapy, when the therapeutic or prophylactic transgene is expressed by the host cells, these cells are often also recognized by the cells of the immune system and cytotoxic T cells may destroy the cells expressing the transgene, thereby limiting the efficacy of gene therapy. If the cells expressing the transgene simultaneously express a CTLA-4BP, this will reduce the activity of those cytotoxic T cells, thereby prolonging the survival of the transduced cells and improving the efficacy of gene therapy.

The present invention additionally provides a method to design or identify small molecule agonists and antagonists that either enhance or inhibit signaling through CD28 and/or CTLA-4 molecules. Methods known to those skilled in the art, such as X-ray crystallography, are used to identify the 3-dimensional structures of proteins (i.e., the CD28BPs and CTLA-4BPs of the invention) and fragments thereof of each. These and other methods can be used to identify and determine the conformations and structures that contribute to the preferential binding of the NMCS molecules (e.g., CD28BPs and CTLA-4BPs) of the invention to CD28 and CTLA-4. Based on the information obtained, small molecules that specifically bind to CD28 or CTLA-4 can be designed. Functional screening assays known to those skilled in the art, such as in vitro T cell proliferation/activation assays, can be used to analyze whether such molecules are specific antagonists or agonists. The resulting small molecules that are agonists for CD28 and/or antagonists for CTLA-4 can be used to, e.g., enhance or modify T cell dependent immune responses. Similarly, small molecules that are antagonists for CD28 and/or agonists for CTLA-4 can be used to, e.g., down-regulate or modify T cell specific immune responses and/or to induce tolerance and/or anergy. These various types of small molecules optionally are beneficial as, e.g., vaccine adjuvants and, e.g., in treating diseases when manipulation of T cell response is desired.

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The invention includes methods of designing or identifying CD28 agonists that enhance or inhibit signaling through either CD28 or CTLA-4 molecules of T-cells, based on visual viewing and/or analysis of the three-dimensional structure (e.g., X-ray crystallography), an analysis of the residues involved in CD28 and/or CTLA-4 binding, and

5   the positions and types of such residues of any of the polypeptides of the invention as found in SEQ ID NOS:48-94, 174-252, 263-272, 283-293, or fragments thereof.

The invention also includes methods of treating a disease or disorder in a subject in need of such treatment, comprising: administering to the subject any NCSM polypeptide described herein in an amount effective to treat said disease or disorder. In

10   another aspect, the invention provides methods for therapeutic or prophylactic treatment of a disease or disorder in a subject in need of such treatment, comprising: administering to the subject any NCSM polypeptide and an immunogen specific for said disease or disorder, wherein the combined amount of polypeptide and immunogen is effective to prophylactically or therapeutically treat said disease or disorder. In some such methods, the

15   polypeptide is present in an amount sufficient to enhance, diminish or modify an immune response induced by the immunogen. The composition may comprise the polypeptide, the immunogen, and a pharmaceutically acceptable excipient is administered to the subject in an amount effective to treat the disease or disorder. For all such methods, the subject may be a mammal, including, e.g. a human. Further, for some such methods, the polypeptide is

20   administered in vivo to the subject or ex vivo to a population of cells obtained from the subject. In another aspect, the invention includes methods for treating a disease or disorder described herein in a subject in need of such treatment, comprising administering to the subject a NCSM polypeptide in an amount effective to treat the disease or disorder.

The invention includes methods of designing or identifying CD28 agonists

25   that enhance or inhibit signaling through either CD28 or CTLA-4 molecules of T-cells, based on visual viewing and/or analysis of the three-dimensional structure (e.g., X-ray crystallography), an analysis of the residues involved in CD28 and/or CTLA-4 binding, and the positions and types of such residues of any of the polypeptides of the invention as found in SEQ ID NOS:48-94, 174-252, 263-272, 283-293, or fragments thereof.

30   The NCSM polynucleotides and polypeptides, B7-1 and B7-2 polynucleotide variants and polypeptide variants, and fragments and homologues of all such molecules as described throughout, can be administered to a subject by any one of the delivery routes described below (including, but not limited to, e.g., intramuscularly, intradermally,

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subdermally, subcutaneously, orally, intraperitoneally, intrathecally, intravenously, mucosally, systemically, parenterally, via inhalation, or placed within a cavity of the body (including, e.g., during surgery)).

INTEGRATED SYSTEMS

5   The present invention provides computers, computer readable media, and integrated systems comprising character strings corresponding to the sequence information herein for the polypeptides and nucleic acids herein, including, e.g., those sequences listed herein and various silent substitutions and conservative substitutions thereof. Various methods and genetic algorithms (GAs) known in the art can be used to detect homology or

10   similarity between different character strings, or can be used to perform other desirable functions such as to control output files, provide the basis for making presentations of information including the sequences and the like. Examples include BLAST, discussed *supra.*

          Thus, different types of homology and similarity of various stringency and

15   length can be detected and recognized in the integrated systems herein. For example, many homology determination methods have been designed for comparative analysis of sequences of biopolymers, for spell-checking in word processing, and for data retrieval from various databases. With an understanding of double-helix pair-wise complement interactions among 4 principal nucleobases in natural polynucleotides, models that simulate

20   annealing of complementary homologous polynucleotide strings can also be used as a foundation of sequence alignment or other operations typically performed on the character strings corresponding to the sequences herein (e.g., word-processing manipulations, construction of figures comprising sequence or subsequence character strings, output tables, etc.). An example of a software package with GAs for calculating sequence similarity is

25   BLAST, which can be adapted to the present invention by inputting character strings corresponding to the sequences herein.

          Similarly, standard desktop applications such as word processing software (e.g., Microsoft Word™ or Corel WordPerfect™) and database software (e.g., spreadsheet software such as Microsoft Excel™, Corel Quattro Pro™, or database programs such as

30   Microsoft Access™ or Paradox™) can be adapted to the present invention by inputting a character string corresponding to the NCSM polypeptides or polynucleotides of the invention or both, or fragments of either. For example, the integrated systems can include

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the foregoing software having the appropriate character string information, e.g., used in conjunction with a user interface (e.g., a GUI in a standard operating system such as a Windows, Macintosh or LINUX system) to manipulate strings of characters. As noted, specialized alignment programs such as BLAST can also be incorporated into the systems

5   of the invention for alignment of nucleic acids or proteins (or corresponding character strings).

Integrated systems for analysis in the present invention typically include a digital computer with GA software for aligning sequences, as well as data sets entered into the software system comprising any of the sequences described herein. The computer can

10   be, e.g., a PC (Intel x86 or Pentium chip- compatible DOS™, OS2™ WINDOWS™ WINDOWS NT™, WINDOWS95™, WINDOWS98™ LINUX based machine, a MACINTOSH™, Power PC, or a UNIX based (e.g., SUN™ work station) machine) or other commercially common computer which is known to one of skill. Software for aligning or otherwise manipulating sequences is available, or can easily be constructed by

15   one of skill using a standard programming language such as Visualbasic, Fortran, Basic, Java, or the like.

Any controller or computer optionally includes a monitor which is often a cathode ray tube ("CRT") display, a flat panel display (e.g., active matrix liquid crystal display, liquid crystal display), or others. Computer circuitry is often placed in a box which

20   includes numerous integrated circuit chips, such as a microprocessor, memory, interface circuits, and others. The box also optionally includes a hard disk drive, a floppy disk drive, a high capacity removable drive such as a writeable CD-ROM, and other common peripheral elements. Inputting devices such as a keyboard or mouse optionally provide for input from a user and for user selection of sequences to be compared or otherwise

25   manipulated in the relevant computer system.

The computer typically includes appropriate software for receiving user instructions, either in the form of user input into a set parameter fields, e.g., in a GUI, or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language

30   for instructing the operation of the fluid direction and transport controller to carry out the desired operation.

The software can also include output elements for controlling nucleic acid synthesis (e.g., based upon a sequence or an alignment of a sequence herein) or other

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operations which occur downstream from an alignment or other operation performed using a character string corresponding to a sequence herein.

The invention provides a computer or computer readable medium comprising a database comprising a sequence record comprising one or more character string

5   corresponding to a nucleic acid or protein sequence selected from SEQ ID NOS:1-272 and 283-293.

In another aspect, the invention provides an integrated system comprising a computer or computer readable medium comprising a database comprising at least one sequence record, each comprising at least one character string corresponding to a nucleic

10   acid or protein sequence selected from SEQ ID NOS:1-272 and 283-293, the integrated system further comprising a user input interface allowing a user to selectively view one or more sequence records. For some such integrated systems, the computer or computer readable medium comprising an alignment instruction set which aligns the character strings with at least one additional character string corresponding to a nucleic acid or protein

15   sequence. The instruction set may comprise one or more of: a local homology comparison determination, a homology alignment determination, a search for similarity determination, and a BLAST determination. Some such systems may further comprise a user readable output element which displays an alignment produced by the alignment instruction set.

In some aspects, the computer or computer readable medium further

20   comprises an instruction set which translates at least one nucleic acid sequence comprising a sequence selected from SEQ ID NOS:1-47, 95-173, and 253-262 into an amino acid sequence. In other aspects, the computer or computer readable medium further comprising an instruction set for reverse-translating at least one amino acid sequence comprising a sequence selected from SEQ ID NOS:48-94, 174-252, 263-272, and 283-293, into a nucleic

25   acid sequence. For some such systems, the instruction set selects the nucleic acid sequence by applying a codon usage instruction set or an instruction set which determines sequence identity to a test nucleic acid sequence.

Also provided is a method of using a computer system to present information pertaining to at least one of a plurality of sequence records stored in a database, each of said

30   sequence records each comprising at least one character string corresponding to SEQ ID NOS:1-272 and 283-293, the method comprising: determining a list of one ore more character strings corresponding to one or more of SEQ ID NOS:1-272 and 283-293, or a subsequence thereof; determining which one ore more character strings of said list are

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selected by a user; and displaying the selected character strings, or aligning the selected character strings with an additional character string. Some such methods further comprise displaying an alignment of the selected character string with the additional character string and/or displaying the list.

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KITS

The present invention also provides kits including the NCSM polypeptides, polynucleotides, expression vectors, cells, vaccines, methods, compositions, systems, and apparatuses of the invention. Kits of the invention optionally comprise at least one of the

10 following of the invention: (1) an apparatus, system, system component, or apparatus component as described herein; (2) at least one kit component comprising a NCSM polypeptide or polynucleotide, soluble NCSM polypeptide or polynucleotide, or fragment thereof; an NCSM-Ig or NCSM-ECD-Ig fusion protein; plasmid expression vector encoding a NCSM polypeptide, soluble NCSM polypeptide, or fragment thereof; cell expressing a

15 NCSM polypeptide, soluble NCSM polypeptide, or fragment thereof; a composition or vaccine composition comprising at least one of any such component; (3) instructions for practicing any method described herein, including a therapeutic or prophylactic methods, instructions for using any component identified in (2) or any vaccine or composition of any such component; and/or instructions for operating any apparatus, system or component

20 described herein; (4) a container for holding said at least one such component or composition, and (5) packaging materials.

In a further aspect, the present invention provides for the use of any apparatus, component, composition, or kit described above and herein, for the practice of any method or assay described herein, and/or for the use of any apparatus, component,

25 composition, or kit to practice any assay or method described herein.

EXAMPLES

The following examples are offered to illustrate the present invention, but not to limit the spirit or scope of the present invention in any way.

30 MATERIALS AND METHODS.

A.   Isolation of Mammalian Parental cDNAs for Library Construction.

Human, rhesus monkey, baboon, orangutan, cow (GenBank Acc. No. Y09950), cat, and rabbit (GenBank Acc. No. D49843) wild-type B7-1 (CD80) parental

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genes were cloned by the reverse transcriptase polymerase chain reaction (RT-PCR) method. RAJI, PUTI, LCL8664, and 26CB-1 cell lines were used as sources of total or messenger RNA (mRNA) for human (Homo sapiens), orangutan (Pongo pygmaeous), rhesus monkey (Macaca mulatta)(GenBank Acc. No. U19840) and baboon (Papio

5   hamadryas) B7-1 genes for B7-1 cDNA preparation. mRNA or total RNA encoding feline (Felis catus), bovine (Bos taurus) and rabbit (Oryctolagus cuniculus sub-species domesticus) B7-1 genes for B7-1 cDNA preparation were obtained from peripheral blood mononuclear cells (PBMCs) derived from the respective species. PBMCs were isolated from cat, cow, and rabbit intravenous blood draws by Ficoll gradient separation. The cells

10 were then activated for 2 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (HyClone, Logan, UT), 5 microgram/milliliter (ug/ml) lipopolysaccharides (LPS), 0.25µg/ml pokeweed mitogen (PWM) and 0.1µg/ml phytohemagglutinin (PHA). Cell lines were maintained at 37°C in DMEM containing 10% fetal calf serum.

15   The cell lines or activated PBMCs were harvested and mRNA or total RNA was isolated using FastTrack 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA) or Promega RNAgents Total RNA Isolation System kit (Promega, Madison, WI), respectively. Primers used to clone the respective mammalian B7-1 cDNAs were designed based on published sequences for human, bovine and rabbit B7-1 genes (see, e.g., Freeman, G.J. et al.

20 (1989) J Immunol 143:2714-22; Parsons, K.R. & Howard, C.J. (1999) Immunogenetics 49:231-4; and Isono, T. & Seto, A. (1995) Immunogenetics 42:217-20)(see also, for human B7-1, GenBank Access. Nos. U33208, AF024703; Cow B7-1, GenBank Acc. No. Y09950; rabbit B7-1, GenBank Access. No. D49843. The primers, which were purchased from Gibco BRL, contained a Bam H I site 5' of the start codon and a Kpn I site 3' of the stop

25 codons. cDNA was generated using the mRNA or total RNA in the Invitrogen cDNA Cycle kit. The cDNAs were generated by RT-PCR, which was performed using the cDNA Cycle kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and standard techniques. Each cDNA was then used as a template for PCR generation of, e.g., double-stranded cDNA using primer(s) specific for each species.

30   All primers used to clone B&-1 (CD80) cDNAs contained a Bam HI site 5' of the species start codon and a Kpn I site 3' of the species stop codons. The PCR products were gel purified using the Bio101 GENECLEAN II kit, digested with BamHI and Kpn I or Asp 718, and ligated into a pcDNA3.1(-) expression vector (Invitrogen, Carlsbad, CA)

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digested with BamHI and Kpn I or Asp 718. The Life Technologies E. coli strain DH10B was transformed with the cDNA clones; colonies were picked, grown, and the clones were isolated from the bacteria using the Qiagen Plasmid Maxi kit (Qiagen, Valencia, CA).

B.   Generation of Recombinant Nucleic Acid Libraries.

5   Recombinant libraries comprising recombinant (chimeric) nucleic acid sequences were generated by recursive sequence recombination procedures using the seven mammalian cDNAs isolated as described above. In one aspect, libraries comprising shuffled chimeric nucleic acid sequences were generated by applying DNA shuffling procedures to the seven mammalian cDNA sequences as described previously in, e.g.,

10   Stemmer, W. (1994) Nature 370:389-391 (1994) and Crameri, A. et al. (1998) Nature 391:288-91, and other references cited above in the section describing recursive sequence recombination and shuffling methods. The shuffled nucleotide sequences were digested with Bam HI and Asp 718 and gel purified using standard techniques. The resulting chimeric shuffled nucleotide sequences were cloned into a pcDNA3.1⁻ expression vector

15   (Invitrogen, Carlsbad, CA) using standard cloning techniques (see, e.g., Sambrook, *supra*) and according to manufacturer's instructions. The vector was used for transfecting cells as described below.

              The FLAG sequence (DYKDDDDK) was inserted at the junction separating the sequence encoding the signal peptide and the sequence encoding the mature polypeptide

20   (e.g., mature coding region) for each of the human B7-1 and CD28BP clones (e.g., CD28BP-15) using the ExSite PCR site-directed mutagenesis kit (Stratagene, San Diego, CA) according to manufacturer's instructions. The nucleotide sequences corresponding to the signal sequence and mature coding region were determined for each shuffled nucleotide sequence by comparison with the known sequences corresponding to the signal sequence

25   and mature coding region for hB7-1. Mutagenesis primers were designed with the FLAG sequence flanked by 24 nucleotides of signal and mature coding sequence specific to each clone. Plasmid DNA was prepared and purified from the cDNA libraries following standard procedure in Maniatis et al., Molecular Cloning: A laboratory Manual, Cold Spring Harbor, NY (1987).

30   C.   Protein Conjugation.

              Soluble CD28-Ig (sCD28-Ig) is a soluble fusion protein between the extracellular domain of human CD28 and the Fc portion of human immunoglobulin G (IgG); soluble CTLA-4-Ig (sCTLA-4-Ig) is a soluble fusion protein between the

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extracellular domain of human CTLA-4 and a human Ig C gamma chain (see, e.g., Linsley et al. (1991), J Exp Med 174:561-569). Soluble CD28-Ig Fc (Fc portion of human IgG1) and soluble CTLA-4-Ig Fc (Fc portion of human IgG1) fusion proteins were both obtained from R&D Systems, Minneapolis, MN. NHS-biotin was obtained from Pierce (Rockford,

5   IL) and Fluorescein Isothiocyanate Isomer I (FITC) was obtained from Molecular Probes (Eugene, OR).

Molar ratios of 1:38.5 for CTLA-4-Ig Fc:FITC and 1:35 for CD28-Ig Fc:Biotin were used during the conjugation. Proteins at 1-3 mg/ml were dialyzed vs. 0.1 M Carbonate buffer for FITC conjugation and 0.1M Sodium Bicarbonate buffer for Biotin

10   conjugation. Ratios of 155 ug FITC/1 mg of CTLA-4-Ig Fc and 124 ug Biotin/1 mg CD28Fc were used during the conjugation. FITC or biotin at 2 mg/ml in dimethyl sulfoxide (DMSO) was added dropwise while vortexing to dialyzed protein, incubated at 25°C in the dark for 2 hours, and then dialyzed against PBS overnight to exchange buffers. (For additional methods, see Linsley et al., *supra.*)

15   D.   Binding Activity Assays and Flow Cytometry.

Soluble CD28-Ig and soluble CTLA-4-Ig fusion proteins were conjugated with biotin and fluorescein isothiocyanate, respectively, as described above. The transfectants were treated with 0.5 mM EDTA in PBS/2% FCS for 15-30 minutes. Several representative wells were counted to determine the number of cells per well. An equal

20   volume of conjugated sCD28-Ig (Fc) or conjugated sCTLA-4-Ig (Fc) was added to the transfected 293 or COS-7 cells at appropriate concentrations as follows. For competitive binding assays, the transfected cells were first incubated with biotin-conjugated sCD28-Ig at room temperature. FITC-conjugated CTLA-4-Ig was then added to this incubation mixture 15 minutes (min) after the addition of biotin-conjugated sCD28-Ig, and the entire mixture

25   was incubated for an additional 1 hour and 45 minutes. (Alternatively, an individual binding assay can be performed using the same procedure, but in which the transfected cells are incubated individually with biotin-conjugated sCD28-Ig or FITC-conjugated CTLA-4-Ig.)

The labeled cells were subsequently handled at 4°C, washed twice with

30   DMEM/10% FCS and incubated with 0.1 µg/ml Streptavidin-phycoerythrin (PE) (Pharmingen, San Diego, CA) in 100 µl for 15 min. Biotin binds the fluorescence marker Streptavidin-PE. (R-PE has an excitation maximum of 565 nanometers (nm) and an emission maximum of 578 nm. B-PE has excitation and emission maxima of 545 nm and

578 nm, respectively. FITC has excitation and emission maxima of 494 and 519, respectively.) The cells were again washed twice and resuspended in 200 µl medium with 5µg/ml propidium iodide (PI). The cells were then analyzed using a FACSCalibur flow cytometer and CellQuest software (BDIS, San Jose, CA). Cell sorting was performed by

5   flow-cytometry based cell sorting screening methods using FACS (Fluorescence-Activated Cell Sorting) Vantage SE cell sorter (BDIS) (Becton Dickinson; San Jose, CA). The staining concentration was determined for each labeled protein to provide a maximal Mean Fluorescence Intensity (MFI) and minimal background signal (*e.g.*, optimum staining concentration was the concentration per 10^6 cells).

10   Representative binding profile for the competitive binding assays are shown in Figures 4A-4D. For the individual binding assay, individual binding profiles are generated for binding of the transfected cells to each of the biotin-conjugated sCD28-Ig or FITC-conjugated CTLA-4-Ig (data not shown).

                For a description of flow cytometry cell sorting methods, which are known

15   in the art, see Current Protocols in Immunology, John Colligan et al., eds., Vols. I-IV (John Wiley & Sons, Inc., 1991 and 2001 Supplement); Sambrook; Rapley and Walker, all *supra*, each of which is incorporated herein by reference in its entirety for all purposes.

E.   Library Sorting/Enrichment.

                Libraries were pre-enriched by FACS sorting for preferential binding to

20   CTLA-4 over CD28 or for preferential binding to CD28 over CTLA-4. Library sorting/enrichment was performed as follows. 293 cells were transfected with a bulk population of recombinant clones from the recombinant libraries, and each transfected library was incubated with both soluble reagents sCD28-Ig and sCTLA-4-Ig (see Section D, above). Transfectants from the CTLA-4-Ig binding biased library were incubated with an

25   optimal concentration of sCD28-Ig and a 10-fold lower concentration of sCTLA-4-Ig than optimal. Transfectants from the CD28-Ig binding biased library were labeled with optimal amounts of both soluble reagents. Cells that preferentially bound CD28 over CTLA-4 were sorted from the CD28-Ig binding biased library transfectants, and cells that preferentially bound CTLA-4 over CD28 were sorted from the CTLA-4-Ig binding biased library

30   transfectants.

                Plasmid was recovered from the sorted cells by lysis with 400 µl Hirt's solution (0.6% sodium dodecyl sulfate (SDS), 10 milliMolar (mM) EDTA pH 8.0) for 0.5 hour, the addition of 100 µl of 0.5 M NaCl to the lysate, and the lysate incubated over night.

The lysate was spun (e.g., centrifuged at 14,000xg for 60 minutes), extracted with equal volume of Phenol/Chloroform, ethanol precipitated, and resuspended in 10 µl TE buffer. The isolated plasmid was used to transform *E. coli* strain DH10B and the transformed cells were plated on LB agar plates. All colonies were harvested and combined and plasmid

5   DNA was isolated using the Qiagen Maxiprep kit.

F.   DNA Purification and Transfections.

E. coli strain DH10B (Life Technologies, Rockville, MD) was transformed with Maxiprep DNA from the libraries comprising recombinant (chimeric) nucleic acid clones generated by DNA shuffling or other recursive sequence recombination procedures,

10   as described above. The transformed cells were plated overnight. Individual colonies were picked from the plated libraries and inoculated into 96-well blocks containing 1.2 ml Terrific Broth-amp (50 µg/ml). Each block was also inoculated with a pcDNA3.1-expression vector (Invitrogen, Carlsbad, CA) (control vector) and human CD80 each in one well. The 96-well plate cultures were grown for 20 hours at 37°C, and plasmid DNA was

15   purified using the Biorobot (Qiagen, Valencia, CA). Cells of either the mammalian cell line 293 or COS-7 (or other cell line f=of interest) were plated in 96-well plates at a density of 2×10^4 cells per well the day prior to transfections. The cells were transfected with plasmids encoding a wild-type B7-1 or chimeric polypeptide using Superfect (Qiagen) or Lipofectamine (Life Technologies) according to the manufacturer's instructions.

20   The following procedure was used for large-scale transfections. Large-scale transfections are typically used in pre-enrichment sorts, and for the generation of therapeutic and/or prophylactic tumor vaccines, including, e.g., composition comprising NCSM polypeptide-expressing tumor cells. Human embryonic kidney 293cells (or alternatively, e.g., monkey COS-7 cells or tumor cells) were transfected with human CD80 (B7-1)

25   plasmid DNA using Life Technologies Lipofectamine and OptiMEM medium. Per 20 cm^2 of plated 293 cells, 3 micrograms (µg) DNA in 200 microliters (µl) OptiMEM were combined with 18 µl Lipofectamine in 200 µl OptiMEM. This mixture was incubated for 15-30 minutes at 25°C, 1.6 milliliters (ml) OptiMEM were added, and 2 ml of this mixture were added per 20 cm^2 of plated 293 cells. Transfections were performed in T25 to T175

30   flasks containing 60-80% confluent 293. Cells were incubated for 5-7 hours in a 37°C humidified incubator containing 5% CO_2. An equal volume of Dulbecco's modified Eagle's medium (DMEM)/20% fetal calf serum (FCS) (HyClone, Logan, UT) was added to the flask and incubated overnight. Cells were trypsinized, replated, and incubated for 24

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hours. Cells were then removed from plastic using EDTA treatment. Cells transfected with, respectively, a control vector, pcDNA3.1- expression vector (Invitrogen, Carlsbad, CA), or plasmid vector encoding human CD80 (B7-1) were counted and aliquoted at 2×10^6 cells/ml.

5   The following procedure was used for high-throughput (HTP) transfections for screening library clones in both T cell and binding assays. For Lipofectamine HTP transfections, plated 293 or COS-7 cells were washed 1x with 200 μl PBS, and 50 μl OptiMEM was added to each well. DNA clone concentrations were normalized to 100 ng/μl ± 33 ng/ul. The DNA preparation was diluted to 5 ng/μl ± 33% in OptiMEM, and 50

10   μl was plated per well in empty 96-well U bottom plates. 50 μl of OptiMEM with Lipofectamine at 0.03 ul/1 μl OptiMEM was added to each well containing diluted DNA preparation. Each well contained 50 ng DNA ± 33% and 0.3 μl Lipofectamine. The mix was incubated for 15 minutes at room temperature, and then 20 μl per well of the mixture was added to each well containing 293 or COS-7 cells in 50 μl OptiMEM. The cells were

15   incubated at 37°C for 5-7 hours, 70 μl DMEM/20% FCS was added to each well and the plates were incubated overnight. The wells were subsequently trypsinized, washed 2x with DMEM/10% FCS, replated in sterile V-bottom plates, and incubated overnight. Alternatively, a Superfect (Qiagen) HTP protocol for transfection, and like transfection protocols, can be used by following the manufacturer's instructions.

20   G.   T Cell Proliferation Assays.

          Peripheral blood was obtained from healthy blood donors as standard buffy coat preparations collected at Stanford Medical School Blood Center (Palo Alto, CA). Peripheral blood mononuclear cells (PBMC) were isolated from human blood by centrifugation over Histopaque-1077 (Sigma, St. Louis, MO) (using Ficol gradient

25   separation).

          T cells were isolated and purified either by staining the cells with anti-human CD2 monoclonal antibodies (mAbs) and sorting for CD2 positive ($CD2^+$) cells using a FACS Vantage SE or removing cells that stained with mAbs specific for CD14, CD20, CD56 and CD94 by magnetic beads (Dynabeads, Dynal, Lake Success, NY); Abs were

30   purchased from Pharmigen (San Diego, CA). Magnetic separation of the T cells using Dynal Dynabeads was performed by first labeling PBMCs with pure monoclonal antibodies against CD14, CD20, CD56 and CD94, then labeling the cells with Sheep anti-mouse Dynabeads. Non-T cells were removed by depleting with a magnet. The purity of the T

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cells was 96-99% when analyzed by staining with anti-CD3 mAbs purchased from Pharmigen (San Diego, CA).

T cell proliferation was measured by ^{3}H-thymidine incorporation. Briefly, 293 cells (or COS-7, or other cells of interest) were transfected as described above for HTP transfections with a plasmid (expression vector) encoding a hB7-1 (or other mammalian B7-1), a chimeric CD28BP or CTLA-4BP polypeptide (i.e., a clone selected from the CD28-Ig/CTLA-4-Ig binding screen assay), or with a control vector lacking the B7-1 or NCSM nucleic acid insert. (The Effectine HTP (Qiagen, Valencia, CA) 96-well transfection method, which can also be used for plasmid transfections, was used to transfect cells with CTLA-4BP selected from Round 1 libraries according to the manufacturer's instructions.) Twenty-four hours after transfection, 5×10^4 purified T cells were cultured in triplicate in the presence of irradiated (5000 rads) transfectants and soluble anti-human CD3 mAbs (5 µg/ml) U -bottom 96-well plates (VWR, West Chester, PA) at 37°C in a humidified atmosphere containing 5% CO_2 in Yssel's medium supplemented with 10% FCS (200 µl/well) for a total of 3 days (72 hours). 1 microCurie (µCi)/well of ^{3}H-thymidine (Amersham, Piscataway, NJ) was added by pulsing to the cell cultures during the last 8 hours of the culture period, and the cells were harvested for counting onto filter paper by a cell harvester (Tomtec, Hamden, CT). Yssel's medium is described in Yssel et al. (1984) J Immunol Methods 72(1):219. ^{3}H-thymidine uptake/incorporation in the cultured cells was determined by measuring the radioactivity on the dried filters using a MicroBeta scintillation counter (Wallac, Turku, Finland). Proliferation of T cells is expressed as the mean counts per minute (cpm) of triplicate wells. The results shown are representative of typically an average of 6 experiments. Chimeric clones that induced or inhibited T cell proliferation at a level about equal to, greater than, or less than that observed with human CD80 (hB7-1) were identified and selected for further characterization.

H.   Mixed Lymphocyte Culture Assay.

Proliferation of purified T cells was also measured in mixed lymphocyte cultures (MLC). Mixed lymphocyte reaction (MLR) was performed using irradiated PBMC as stimulator cells and allogeneic PBMC as responders. Stimulator cells were irradiated (2500 rads) and co-cultured with allogeneic PBMC (1×10^5 cells/well) in 96-well flat-bottomed microtiter culture plates (VWR) at 1:1 ratio for a total of 5 days. During the last 8 hours of the culture period, the cells were pulsed with 1uCi/well of ^{3}H-thymidine, and the cells were harvested for counting onto filter paper by a cell harvester as described above.

[3]H-thymidine incorporation was measured as described above for purified T cells. Proliferation of T cells was expressed as the mean cpm of triplicate wells. The results shown representative of more than one experiment.

I.   Analysis of Cytokine Levels in Culture Supernatants.

5   Supernatants of cell cultures from mixed lymphocyte reaction were collected after 48 hours and stored at -80°C until they were analyzed for the presence of various cytokines. IL-10 and IFN-gamma levels were determined in duplicate using cytokine-specific ELISA kits (R&D Systems, Minneapolis, MN) by following the manufacturer's instructions (R&D Systems, Minneapolis, MN). Controls were provided in the kits.

10   J.   Concentration-Dependent CTLA4-Ig or CD28-Ig Binding Assays.

For each particular transfectant, 2 x 10^{5} cells were incubated with serial dilutions of CTLA-4-Ig in phosphate-buffered saline (PBS) containing 5% FCS for 30 minutes on ice. Next, the cells were washed with PBS-FCS and incubated subsequently with a saturating concentration of FITC-conjugated goat anti-human IgG mAb (Fc specific)

15   (Caltag Laboratories, San Francisco, CA) for another 30 minutes on ice. The cells were analyzed using a FACSCalibur flow cytometer and CellQuest software, and cell sorting was performed using FACS Vantage SE cell sorter (BDIS) described above. Plasmids were recovered from the sorted cells by Hirt preparation as described above.

20   EXAMPLE I: Cloning of Parent cDNA Sequences for Library Construction

The cDNAs encoding human, rhesus monkey, orangutan, baboon, bovine, rabbit, and feline B7-1 (CD80) co-stimulatory molecules were cloned by RT-PCR. These starting B7-1 genes encoded polypeptide molecules with, by comparison, amino acid sequence identities ranging from about 58-98% amino acid sequence identity using Jotun

25   Hein method, DNASTAR (in MegaLine™ DNASTAR package, MegaLine™ Ver. 4.03), following manufacturer's instructions and using default values specified in the program. The polynucleotide sequences for baboon B7-1 (SEQ ID NO:46) and orangutan B7-1 (SEQ ID NO:47) are examples of WT NCSM polynucleotides whose sequences were previously unknown. These baboon B7-1 and orangutan B7-1 polynucleotides, as well as homologues

30   and baboon B7-1 (SEQ ID NO:93) and orangutan B7-1 (SEQ ID NO:94) polypeptides encoded therefrom, are included as NCSM molecules of the invention.

The RAJI, PUTI, LCL8664, and 26CB-1 cell lines were used as sources of messenger or total RNA for primate B7-1 cDNA preparation as described previously.

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Intravenous draws of peripheral blood were used as the source of messenger or total RNA for cat, cow, and rabbit B7-1 cDNA preparation as discussed previously. Peripheral blood mononuclear cells (PBMCs) were isolated from the cat, cow, and rabbit blood draws by Ficol gradient separation.

5 PBMCs were activated for 2 days with medium containing lipopoly-saccharide (LPS), pokeweed mitogen (PWM) and phytohemagglutinin (PHA). Cell lines and activated PBMCs were harvested and mRNA or total RNA was isolated. cDNA was generated from messenger or total RNA by using the Invitrogen cDNA Cycle kit. By using primers specific for each species, double-stranded cDNA was generated via PCR.

10

EXAMPLE II: Preparation and Screening of Round I NCSM Libraries

Nucleic acid libraries comprising recombinant nucleic acid sequences were generated by using the seven cloned cDNA wild-type CD80 nucleic acid sequences as parental sequences and applying recursive sequence recombination methods as described
15 above to such sequences. In one aspect, libraries comprising chimeric nucleic acid sequences were generated by applying DNA shuffling procedures to the seven mammalian cDNA sequences as described previously in, e.g., Stemmer, W. (1994) Nature 370:389-391 (1994) and Crameri, A. et al. (1998) Nature 391:288-91, each of which is incorporated herein by reference in its entirety for all purposes. Sequencing of randomly selected
20 chimeric clones a recombinant library indicated that 12 out of 12 clones comprised nucleotide fragments from at least two of the starting genes, illustrating efficient chimerism.

Initial screening of the resulting chimeric NCSM clones was based on binding assays in which binding of a polypeptide encoded by a clone nucleic acid and expressed on the surface of a cell transfected for one of the two B7-1 ligands, CD28 or
25 CTLA-4, was evaluated. For a detailed review of the binding assays, see the "Materials and Methods" section above. In brief, cells from a human HEK 293 cell line were transfected with plasmid DNA of the resulting recombinant libraries of recursively recombined NCSM polynucleotides. Library transfectants were incubated with soluble CD28-Ig and CTLA-4-Ig conjugated with fluorescence-indicators, and sorted according to their fluorescence via
30 FACS-sorting and 96-well format HTP transfections. The invention is not limited by the choice of fluorescence indicator molecules used (i.e., numerous indicator molecules may be used).

Flow cytometry-based cell sorting was used to screen the libraries for clones with increased or decreased relative binding to CD28 and CTLA-4. (Fluorescently-labeled soluble CD28-Ig and CTLA-4-Ig molecules were used as soluble CD28 and CTLA-4 receptors, respectively, in the competitive or individual binding assays.) Transfected cells

5   that preferentially bound CD28 over CTLA-4 (as compared to CD28 and CTLA-4 binding of wild-type co-stimulatory B7-1 molecules, such as, e.g., hB7-1) in an individual or competitive binding assay were sorted out from the library transfectants as were cells that preferentially bound CTLA-4 over CD28 (again, as compared to CD28 and CTLA-4 binding of wild-type B7-1 molecules, such as, e.g., hB7-1) in an individual or competitive

10   binding assay. A large fraction of the cell-surface displayed shuffled chimeric molecules displayed exhibited binding to either sCD28-Ig or sCTLA-4-Ig. Less than 10% of the randomly selected chimeras demonstrated no binding to either sCD28-Ig or sCTLA-4-Ig (data not shown), indicating high functional fitness of polypeptides and proteins generated by DNA shuffling of natural wild-type mammalian B7-1 genes.

15   Plasmid DNA encoding the recursively recombined NCSM molecules was recovered from both categories of sorted cells and DNA was prepared for binding assays. In the binding assays, DNA from individual clones was transfected into human HEK 293 cells (or COS or other cells of interest), and the cells were analyzed for the ability to preferentially bind either fluorescent-labeled sCD28-Ig or fluorescent-labeled sCTLA-4-Ig

20   (either separately or competitively), as compared to the binding of cells expressing wild-type B7-1 to the labeled sCD28-Ig or sCTLA-4-Ig. Clones exhibiting preferential binding for either CD28 or CTLA, as compared to the binding of wild-type (WT) B7-1 to CD28 or CTLA-4, were then analyzed by DNA sequencing, amino acid sequencing, and functional assays as described below.

25   Subsequent analysis of 1000 individual clones recovered from the sorted cells identified a number of clones with altered ligand binding profiles for CD28 and CTLA-4 relative to the binding of wild-type B7-1 to CD28 and CTLA-4. Four clones with preferential binding to sCD28-Ig over sCTLA-4-Ig (compared to the relative binding of WT hB7-1 to sCD28-Ig and sCTLA-4-Ig) were subjected to more detailed analysis; reduced

30   binding of these clones to sCTLA-4-Ig was observed in at least two separate experiments, while their binding to sCD28-Ig remained intact (data not shown). Although the level of binding of these clones to sCTLA-4-Ig was reduced, however, it was still detectable (data not shown). Preferential binding to sCTLA-4-Ig over sCD28-Ig (compared to the relative

binding of WT hB7-1 to sCD28-Ig and sCTLA-4-Ig) was observed in at least 15 clones of the 1000 individual Round 1 clones analyzed by flow cytometry (data not shown). Again, the loss of binding to sCD28-Ig was only partial.

Based on the results of this screening assay, exemplary NCSM molecules having a desired phenotype comprising an altered CD28/CTLA-4 binding affinity ratio or CTLA-4/CD28 binding affinity ratio (relative to that of WT hB7-1) were identified. Plasmid DNA encoding NCSM molecules of these selected clones was recovered and DNA was prepared for subsequent binding and T cell proliferation functional assays.

In T cell proliferation assays, DNA from each of the selected exemplary CD28BP or CTLA44BP clones identified in the binding assays was transfected into either monkey (COS-7) or human embryonic kidney (human 293 cell line) cells *in vitro*, using procedures described above, and tested for an ability to induce or inhibit T cell proliferation as compared to human wild-type B7-1. Anti-CD28 mAbs can be used as a positive control. (An alternative functional test is to transfect or vaccinate human or animal cells *in vivo* with the nucleic acid from a selected clone, as described below.) To stimulate T cell activation via two-signaling pathway, human T cells were incubated with the cells expressing a selected NCSM molecule and anti-CD3 antibody. See "T Cell Proliferation Assays" in "Materials and Methods." For example, by incubating T cells with anti-CD3 antibody and cells expressing a CD28BP of the invention, the cell surface-expressed CD28BP bound CD28 receptor on the T cells and the anti-CD3 bound the CD3 T cell receptor. This was followed by addition of H^3-thymidine to measure the increase in DNA synthesis (a commonly used indication of cell proliferation), as described previously. Figures 10 and 12 illustrate results of T cell proliferation assays.

Clones that induced T cell proliferation that was about equal to, equal to or higher than that induced by WT hB7-1 were identified and selected for further characterization from the recombinant nucleic acid library pre-enriched by FACS sorting for clones having preferential binding to CD28 over CTLA-4. Four exemplary clones having the most biased binding to CD28 and an ability to induce a cell proliferation response about equal to or higher than that induced by WT hB7-1 were identified and designated Round 1 (R1) CD28BP-71, -84, -118, and -126 clones. Amino acid and nucleic acid sequences of these NCSM clones were determined using standard sequencing techniques described above.

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Clones that reduced or suppressed T cell proliferation relative to the that generated by WT human B7-1 were identified and selected for further characterization from the recombinant nucleic acid library pre-enriched by FACS sorting for clones having preferential binding to CTLA-4 over CD28. Five exemplary clones exhibiting the most

5   biased binding to CTLA-4 and the ability to reduce or suppress a T cell proliferation relative to that generated by WT human B7-1 were identified and designated R1 CTLA-4BP-5, -7, -11, -13, and -27 clones. The amino acid and nucleic acid sequences of these NCSM clones were determined using standard sequence techniques as described above.

Round 1 clones showed diverse amino acid and nucleotide differences from

10   wild-type B7-1 sequences. For example, R1 CTLA4BP clones demonstrated a 93.4-97.6% amino acid identity with a wild-type B7-1 molecule and a 97-98.5% identity at the nucleotide level. In competitive binding assays using CD28-Ig and CTLA-4-Ig, the R1 CTLA-4BPs displayed preferential binding to CTLA-4-Ig as compared to the wild-type B7-1 binding preference to CD28-Ig and CTLA-4-Ig.

15

EXAMPLE III: Preparation and Screening of Round 2 NCSM Libraries
        The nucleotide sequences (or nucleotide segments or fragments thereof) corresponding to R1 CD28BP-71, -84, -118, and -126 clones were used as parental sequences for recursive sequence recombination in the generation of a second round

20   CD28BP NCSM recombinant nucleic acid library. The nucleotide sequences (or nucleotide segments or fragments thereof) corresponding to R1 CTLA-4BP-5, -7, -11, -13, and -27 clones were used as parental sequences for DNA shuffling in the generation of a second round CTLA-4BP NCSM recombinant nucleic acid library. As in Round 1, the nucleotide sequences corresponding to the selected CD28BP clones were recursively recombined with

25   one another and the selected CTLA-4BPs were recursively recombined with one another by DNA shuffling in parallel experiments. Two separate sets of libraries were generated (one derived from the selected CD28BP parental clones and one derived from the selected CTLA-4BP clones). Binding assays were performed as previously described on the recombinant libraries generated in Round 2 (e.g., libraries containing recursive sequence

30   recombinants were incubated with soluble CD28-Ig and CTLA-4-Ig conjugated with fluorescence indicators, and sorted according to their fluorescence binding profiles).

Screening of 1000 individual clones from both libraries identified a number of clones that exhibited strongly biased binding to either sCD28-Ig or sCTLA-4-Ig. The second round of breeding resulted in a number of different clones exhibiting biased (altered)

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binding to sCD28-Ig or sCTLA-4-Ig, respectively. For example, a number of Round 2 (R2) CD28BP clones showed even greater preferential binding to CD28 than did the R1 CD28BP clones. Similarly, a number of R2 CTLA-4BP clones showed even greater preferential binding to CTLA-4 than did the R1 CTLA-4BP clones. From the shuffled recombinant

5   libraries of the second round of breeding, a number of clones with further biased binding to sCD28-Ig or sCTLA-4-Ig, respectively, as compared to the clones selected from the first round of breeding (data not shown), were selected from the Round 2 libraries using the same criteria as in Round 1. The clones displayed a range of expression on the stable transfectants as compared to WT hB7-1 transfectants. The plasmid DNA for selected

10   clones was recovered and DNA prepared for further binding and functional assays. In addition, the amino acid and nucleic acid sequences of these clones were determined using standard procedures known in the art.

A.   Round 2 Clones With Preferential Binding Properties.

Seventeen R2 CD28BP clones were found to have preferential binding to

15   CD28 over CTLA-4 as shown in both individual and competitive binding assays between these cells transfected with these clones and soluble CD28-Ig fusions and/or CTLA4-Ig fusions. Binding profiles for the 17 clones are shown in Figure 5. These clones were designated clones Round 2 (R2) CD28BP-1 through CD28BP-17 and their respective amino acid and nucleic acid sequences were determined (see Table 3). Thirty-six R2 CD28BP

20   clones were also found to have preferential binding to CD28 over CTLA-4 relative to WT hB7-1 as demonstrated in both individual and competitive binding assays between these cells transfected with these clones and soluble CD28-Ig fusions and/or CTLA4-Ig fusions. These clones were designated clones R2 CD28A12-5 through CD28E2-4 and their respective amino acid and nucleic acid sequences were determined (see Table 3). Twelve

25   R2 CD28BP clones binding profiles similar to that of WT hB7-1 were also selected from both R2 libraries and their respective amino acid and nucleic acid sequences were determined. Binding profiles for other recombinant CD28BP clones described herein were also generated (data not shown).

Table 3

Nucleic acid SEQ ID NO:	Protein SEQ ID NO:	Clone ID	Binding Score
SEQ ID NO:1	SEQ ID NO:48	R1clone71	1
SEQ ID NO:2	SEQ ID NO:49	R1clone 84	1
SEQ ID NO:3	SEQ ID NO:50	R1clone 118	1

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Nucleic acid SEQ ID NO:	Protein SEQ ID NO:	Clone ID	Binding Score
SEQ ID NO:4	SEQ ID NO:51	R1clone 126	1
SEQ ID NO:5	SEQ ID NO:52	CD28BP1	2
SEQ ID NO:6	SEQ ID NO:53	CD28BP2	2
SEQ ID NO:7	SEQ ID NO:54	CD28BP3	2
SEQ ID NO:8	SEQ ID NO:55	CD28BP4	2
SEQ ID NO:9	SEQ ID NO:56	CD28BP5	2
SEQ ID NO:10	SEQ ID NO:57	CD28BP6	2
SEQ ID NO:11	SEQ ID NO:58	CD28BP7	2
SEQ ID NO:12	SEQ ID NO:59	CD28BP8	2
SEQ ID NO:13	SEQ ID NO:60	CD28BP9	2
SEQ ID NO:14	SEQ ID NO:61	CD28BP10	2
SEQ ID NO:15	SEQ ID NO:62	CD28BP11	2
SEQ ID NO:16	SEQ ID NO:63	CD28BP12	2
SEQ ID NO:17	SEQ ID NO:64	CD28BP13	2
SEQ ID NO:18	SEQ ID NO:65	CD28BP14	2
SEQ ID NO:19	SEQ ID NO:66	CD28BP15	2
SEQ ID NO:20	SEQ ID NO:67	CD28BP16	2
SEQ ID NO:21	SEQ ID NO:68	CD28BP17	2
SEQ ID NO:95	SEQ ID NO:174	CD28A12-5	1
SEQ ID NO:96	SEQ ID NO:175	CD28A4-5*	1
SEQ ID NO:97	SEQ ID NO:176	CD28A4-9	1
SEQ ID NO:98	SEQ ID NO:177	CD28A6-9	1
SEQ ID NO:99	SEQ ID NO:178	CD28A6-1	1
SEQ ID NO:100	SEQ ID NO:179	CD28A8-4	1
SEQ ID NO:101	SEQ ID NO:180	CD28A8-6	1
SEQ ID NO:102	SEQ ID NO:181	CD28B2-8	1
SEQ ID NO:103	SEQ ID NO:182	CD28B4-3	1
SEQ ID NO:104	SEQ ID NO:183	CD28B6-3	1
SEQ ID NO:105	SEQ ID NO:184	CD28B6-6	1
SEQ ID NO:106	SEQ ID NO:185	CD28B8-5*	1
SEQ ID NO:107	SEQ ID NO:186	CD28C11-5	2
SEQ ID NO:108	SEQ ID NO:187	CD28C6-1	1
SEQ ID NO:109	SEQ ID NO:188	CD28C7-3	1
SEQ ID NO:110	SEQ ID NO:189	CD28C8-6	2
SEQ ID NO:111	SEQ ID NO:190	CD28C9-5*	1
SEQ ID NO:112	SEQ ID NO:191	CD28C2-4	1
SEQ ID NO:113	SEQ ID NO:192	CD28D2-3	1
SEQ ID NO:114	SEQ ID NO:193	CD28D2-9	1
SEQ ID NO:115	SEQ ID NO:194	CD28D8-9	1
SEQ ID NO:116	SEQ ID NO:195	CD28D11-1	2
SEQ ID NO:117	SEQ ID NO:196	CD28D12-5	2
SEQ ID NO:118	SEQ ID NO:197	CD28E10-6	1
SEQ ID NO:119	SEQ ID NO:198	CD28F7-2	2
SEQ ID NO:120	SEQ ID NO:199	CD28F8-4	1
SEQ ID NO:121	SEQ ID NO:200	CD28F10-2	1

Nucleic acid SEQ ID NO:	Protein SEQ ID NO:	Clone ID	Binding Score
SEQ ID NO:122	SEQ ID NO:201	CD28F12-5*	1
SEQ ID NO:123	SEQ ID NO:202	CD28G2-8	1
SEQ ID NO:124	SEQ ID NO:203	CD28G1-5	1
SEQ ID NO:125	SEQ ID NO:204	CD28G1-9	1
SEQ ID NO:126	SEQ ID NO:205	CD28H4-3	1
SEQ ID NO:127	SEQ ID NO:206	CD28H11-3	1
SEQ ID NO:128	SEQ ID NO:207	CD28H6-6	1
SEQ ID NO:129	SEQ ID NO:208	CD28E2-4	1
SEQ ID NO:130	SEQ ID NO:209	CD28B4-5a	1
SEQ ID NO:131	SEQ ID NO:210	CD28A2-5	0
SEQ ID NO:132	SEQ ID NO:211	CD28B4-5*	0
SEQ ID NO:133	SEQ ID NO:212	CD28D5-6	0
SEQ ID NO:134	SEQ ID NO:213	CD28D10-4	0
SEQ ID NO:135	SEQ ID NO:214	CD28E2-5*	0
SEQ ID NO:136	SEQ ID NO:215	CD28E5-2	0
SEQ ID NO:137	SEQ ID NO:216	CD28E8-6	0
SEQ ID NO:138	SEQ ID NO:217	CD28E9-6	0
SEQ ID NO:139	SEQ ID NO:218	CD28F3-1	0
SEQ ID NO:140	SEQ ID NO:219	CD28F3-5	0
SEQ ID NO:141	SEQ ID NO:220	CD28F3-6	0
SEQ ID NO:142	SEQ ID NO:221	CD28F11-8	0

Table 3 above presents a summary of the relative binding activities of these selected R2 CD28BP NCSM clones based on three exemplary binding profiles shown in Figures 6B(1)-6B(3). In the three exemplary binding profiles, the Y-axis represents binding to CD28, and the X-axis represents binding to CTLA-4. An exemplary binding profile for the binding of WT B7-1 to CD28 and CTLA-4 is shown in Fig. 6B(1), indicating approximately equal binding affinity of WT B7-1 to CD28 and CTLA-4. An example of a binding profile indicating high preferential binding to CD28 over CTLA-4 relative to that of WT B7-1 is shown in Fig. 6B(3); that is, the clone has a CD28/CTLA-4 binding affinity ratio significantly greater than the CD28/CTLA-4 binding affinity ratio of WT hB7-1. An example of a binding profile indicating intermediate preferential binding to CD28 over CTLA-4 relative to that of WT B7-1 is shown in Fig. 6B(2) (i.e., a CD28/CTLA-4 binding affinity ratio greater than the CD28/CTLA-4 binding affinity ratio of WT hB7-1).

A score is assigned to each clone based upon comparison to the three exemplary binding profiles. A score of zero (0) indicates the CD28BP clone has a binding profile equivalent or substantially equivalent to that of WT B7-1. A score of 1 indicates the CD28BP clone has a binding profile similar to that shown in Fig. 6B(2) (i.e., CD28/CTLA-

4 binding affinity ratio greater than the CD28/CTLA-4 binding affinity ratio of WT hB7-1).

A score of 2 indicates the clone has a binding profile similar to that shown in Fig. 6B(3)

(i.e., a CD28/CTLA-4 binding affinity ratio significantly greater than the CD28/CTLA-4

binding affinity ratio of WT hB7-1). Table 3 shows the clone identification (ID) name for

each selected R2 CD28BP clone and its score.

R2 CD28BP clones 3, 6, and 9 (corresponding to nucleic acid sequences

SEQ ID NOS:7, 10, and 13, and amino acid sequences 54, 57, and 60, respectively)

comprise identical amino acid and nucleic acid sequences; the competitive binding assays

and T cell proliferation assays for these clones were conducted in a repeated manner to

verify functional activity. Clones Round 2 CD28BP-1 and -12 comprise identical amino

acid sequences (amino acid sequences SEQ ID NOS:52 and 63, respectively); however, the

nucleic acid sequences of clones R2 CD28BP-1 and -12 (nucleic acid sequences SEQ ID

NOS:5 and 16, respectively) differ from one another by one nucleic acid residue at position

894 in both sequences. Clone 1 has nucleic acid residue C at position 894 (with the

resulting codon TCC encoding Ser); clone 12 has nucleic acid residue T at position 894

(with the resulting codon TCT also encoding Ser).

Fifty R2 CTLA-4BP clones were found to have preferential binding to CD28

over CTLA-4 as shown in both individual and competitive binding assays between cells

transfected with these clones and fluorescently labeled soluble CD28-Ig fusions and/or

CTLA4-Ig fusions. Exemplary binding profiles for selected clones are shown in Figures

7A-7H. The respective amino acid and nucleic acid sequences of the clones were

determined (Table 4). Table 4 presents a summary of the relative binding activities of these

selected 50 R2 CTLA-4BP clones based on the three exemplary binding profiles shown in

Figs. 6A(1)-6A(3). In the three exemplary competitive binding profiles, the Y-axis

represents binding to CD28, and the X axis represents binding to CTLA-4 (see binding

assays described in "Materials and Methods"). An exemplary binding profile for the

binding of WT B7-1 to CD28 and CTLA-4 is shown in Fig. 6A(1), indicating

approximately equal binding affinity of WT B7-1 to CD28 and CTLA-4. An exemplary

binding profile indicating for a particular clone a preferential binding to CTLA-4 over

CD28 relative to that of WT B7-1 is shown in Fig. 6A(3); the clone has a CTLA4/CD28

binding affinity ratio significantly greater than the CTLA-4/CD28 binding affinity ratio of

WT hB7-1. An exemplary binding profile indicating intermediate preferential binding to

CD28 over CTLA-4 relative to that of WT B7-1 is shown in Fig. 6A(2); the clone has a CTLA4/CD28 binding affinity ratio greater than that of WT hB7-1.

A score is assigned to each NCSM clone based upon comparison to the three exemplary binding profiles. A score of zero (0) indicates the CTLA-4BP clone has a binding profile similar or equivalent to that of WT B7-1. A score of 1 indicates the CTLA-4BP clone has a binding profile similar or equivalent to that shown in Fig. 6A(2) (i.e., with a CTLA-4/CD28 binding affinity ratio greater than the CTLA-4/CCD28 binding affinity ratio of WT hB7-1). A score of 2 indicates the CTLA-4BP clone has a binding profile similar or equivalent to that shown in Fig. 6A(3) (i.e., with a CTLA-4/CD28 binding affinity ratio significantly greater than that of WT hB7-1). The clone identification (ID) name and score assigned to each selected CTLA-4BP clone are shown in Table 4. Binding profiles for other CTLA-4BP clones described herein were also generated (data not shown).

Table 4

Nucleic acid SEQ ID NO:	Protein SEQ ID NO:	Clone ID	Binding Score
SEQ ID NO:22	SEQ ID NO:69	R1-5	2
SEQ ID NO:23	SEQ ID NO:70	R1-7	1
SEQ ID NO:24	SEQ ID NO:71	R1-11	1
SEQ ID NO:25	SEQ ID NO:72	R1-13	1
SEQ ID NO:26	SEQ ID NO:73	R1-27	1
SEQ ID NO:27	SEQ ID NO:74	5x2-10c	2
SEQ ID NO:28	SEQ ID NO:75	5x2-11d	2
SEQ ID NO:29	SEQ ID NO:76	5X2-12F	1
SEQ ID NO:30	SEQ ID NO:77	5x2-2g	2
SEQ ID NO:31	SEQ ID NO:78	5x2-3c	2
SEQ ID NO:32	SEQ ID NO:79	5x2-4c	1
SEQ ID NO:33	SEQ ID NO:80	5x2-7b	1
SEQ ID NO:34	SEQ ID NO:81	5x2-8c	2
SEQ ID NO:35	SEQ ID NO:82	5x3-10e	2
SEQ ID NO:36	SEQ ID NO:83	5X3-11B	1
SEQ ID NO:37	SEQ ID NO:84	5x3-6f	2
SEQ ID NO:38	SEQ ID NO:85	5X4-11D	2
SEQ ID NO:39	SEQ ID NO:86	5X4-12C	2
SEQ ID NO:40	SEQ ID NO:87	5x4-1F	2
SEQ ID NO:41	SEQ ID NO:88	5X5-2E	2
SEQ ID NO:42	SEQ ID NO:89	5X5-6E	2
SEQ ID NO:43	SEQ ID NO:90	5X6-9D	2
SEQ ID NO:44	SEQ ID NO:91	5X8-1F	2
SEQ ID NO:45	SEQ ID NO:92	5X9-12C	2
SEQ ID NO:143	SEQ ID NO:222	5x9-d10	1
SEQ ID NO:144	SEQ ID NO:223	5x6-f6	1

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Nucleic acid SEQ ID NO:	Protein SEQ ID NO:	Clone ID	Binding Score
SEQ ID NO:145	SEQ ID NO:224	5x5-h12	1
SEQ ID NO:146	SEQ ID NO:225	5x5-c10	1
SEQ ID NO:147	SEQ ID NO:226	5x3-e8	1
SEQ ID NO:148	SEQ ID NO:227	5x3-c4	1
SEQ ID NO:149	SEQ ID NO:228	5x3-c3	1
SEQ ID NO:150	SEQ ID NO:229	5x2-h11	1
SEQ ID NO:151	SEQ ID NO:230	5x2-d7	1
SEQ ID NO:152	SEQ ID NO:231	5x2-b7	1
SEQ ID NO:153	SEQ ID NO:232	5x2-b1	1
SEQ ID NO:154	SEQ ID NO:233	5x1-f1	1
SEQ ID NO:155	SEQ ID NO:234	5x1-d7	1
SEQ ID NO:156	SEQ ID NO:235	2x4-g9	1
SEQ ID NO:157	SEQ ID NO:236	2x4-a6	1
SEQ ID NO:158	SEQ ID NO:237	2x2-f3	1
SEQ ID NO:159	SEQ ID NO:238	2x2-f12	1
SEQ ID NO:160	SEQ ID NO:239	2x1-g8	1
SEQ ID NO:161	SEQ ID NO:240	2x1-f10	1
SEQ ID NO:162	SEQ ID NO:241	2x1-c9	1
SEQ ID NO:163	SEQ ID NO:242	2x1-h12	1
SEQ ID NO:164	SEQ ID NO:243	2x1-e2	1
SEQ ID NO:165	SEQ ID NO:244	2x1-c4	1
SEQ ID NO:166	SEQ ID NO:245	2x1-b12	1
SEQ ID NO:167	SEQ ID NO:246	2x2-f1	1
SEQ ID NO:168	SEQ ID NO:247	5X4-h1	2
SEQ ID NO:169	SEQ ID NO:248	5x4-a1	0
SEQ ID NO:170	SEQ ID NO:249	5x2-f3	0
SEQ ID NO:171	SEQ ID NO:250	5x2-e12	0
SEQ ID NO:172	SEQ ID NO:251	2x4-h11	0
SEQ ID NO:173	SEQ ID NO:252	2x3-h2	0
SEQ ID NO:253	SEQ ID NO:263	A-H3-6	1
SEQ ID NO:254	SEQ ID NO:264	A-B11-5	1
SEQ ID NO:255	SEQ ID NO:265	A-E2-6	1
SEQ ID NO:256	SEQ ID NO:266	A-F1-6	1
SEQ ID NO:257	SEQ ID NO:267	A-F6-9	1
SEQ ID NO:258	SEQ ID NO:268	A-H4-5*	1
SEQ ID NO:259	SEQ ID NO:269	A-B4-6	1
SEQ ID NO:260	SEQ ID NO:270	A-F10-1	1
SEQ ID NO:261	SEQ ID NO:271	A-G8-1	1
SEQ ID NO:262	SEQ ID NO:272	A-C9-9	0

The plasmids corresponding to the 36 clones displaying preferential binding to CD28 over CTLA-4 relative to that of WT hB7-1 were recovered; each corresponding CD28BP molecule was recovered, and nucleic acid and amino acid sequences were

determined. The clones were assigned scores of 2 and 1, as shown in Table 3, depending upon the magnitude of the observed preferential binding.

The plasmids for the 12 R2 clones from the CD28BP R2 library that displayed CD28 and CTLA-4 binding profiles equal or approximately equivalent to that of

5   WT B7-1 were recovered; each corresponding clone molecule was recovered and its nucleic acid and amino acid sequences were determined. These clones were assigned a score of zero, as shown in Table 3.

Two groups of R2 CTLA-4BP clones (19 in the first group, Group I, and 26 in the second group, Group II) were identified as having preferential binding to CTLA-4

10   over CD28 relative to that of hB7-1 as demonstrated in competitive binding assays between cells transfected with these clones and fluorescently labeled soluble CD28-Ig fusions and/or CTLA4-Ig fusions. These clones are identified in Table 4, which indicates the clone ID name and binding score (as explained with regard to Table 3); the amino acid and nucleic acid sequences for each CTLA-4BP clone were determined. In general, the R2 clones

15   displayed a greater degree of CTLA-4 binding preference than the R1 clones. In addition, five R2 clones from the CTLA-4 R2 library with binding profiles equal or approximately equivalent to that of WT human B7-1 were identified. Plasmids for all such clones were recovered; the nucleic acid and amino acid sequences were determined for each clone, and each clone was assigned a binding score (see Table 4).

20   Preferred or enhanced binding of a selected clone to CD28 over CTLA-4 relative to hB7-1 was observed by an increase in the observed CD28/CTLA-4 binding affinity ratio for the clone compared to the CD28/CTLA-4 binding affinity ratio of hB7-1. Preferred or enhanced binding of a selected clone to CTLA-4 over CD28 relative to hB7-1 is observed by an increase in the observed CTLA-4/CD28 binding affinity ratio for the

25   clone compared to the CTLA-4/CD28 binding affinity ratio of hB7-1.

The R2 selected clone that exhibited the greatest increase in CD28/CTLA-4 binding affinity ratio compared to the CD28/CTLA-4 binding affinity ratio of hB7-1 was clone R2 CD28BP-15. The identified clone that exhibited the greatest increase in CTLA-4/CD28 binding affinity ratio compared to the CTLA-4/CD28 binding affinity ratio of hB7-

30   1 was clone R2 CTLA-4BP 5x4-12c. Figures 4A-4D show a characterization of competitive ligand binding properties of CD28BP-15 and CTLA-4BP 5x4-12c compared to human B7-1. 293 cells transfected with CD28BP-15, CTLA-4BP 5x4-12c, hB7-1, or a negative control vector (which did not contain the B7-1, CTLA4-BP, or CD28BP nucleotide

<div align="center">215</div>

sequence insert) were stained with fluorescently labeled biotin-conjugated sCD28-Ig or FITC-conjugated CTLA-4-Ig fusion proteins. The results of a representative flow cytometry analysis are shown in Figs. 4A-4D; similar results were obtained in five other experiments. Transfectants expressing CD28BP-15 or CTLA-4BP 5x4-12c, and stained

5   under identical conditions, demonstrated dramatically altered ligand binding profiles, as compared to transfectants expressing hB7-1 stained in an identical manner. In particular, CD28BP-15 showed a significantly changed binding profile to CD28 as compared to the binding of hB7-1 to CD28. CD28BP-15 also showed a significant loss of CTLA-4 binding as compared to the binding of human B7-1 to CTLA-4.

10   Figures 8A-8B show the respective amino acid sequences for CD28BP-12 and CTLA-4BP 5x4-12c and the genealogy of these sequences. The nucleotide and amino acid sequences for each of CD28BP-12 and CTLA-4BP 5x4-12c were aligned with the starting genes to identify the parental origins of the recombinant sequences. The chimeric nature of each recombinant amino acid sequence is indicated by a solid labeled line

15 designating each amino acid subsequence derived from a particular parental species sequence. Any amino acid residue that differs from a residue in the WT human B7-1 sequence in the corresponding (equivalent) amino acid residue position is indicated with a star (*). Three point mutations in CTLA-4BP 5x4-12c that were not derived from any of the starting parental genes are indicated with a solid triangle. The predicted transmembrane

20 domain is illustrated with a dashed line (prediction based on equivalent analysis for mammalian B7-1 molecules in Parsons, K.R. & Howard, C.J. (1999) Immunogenetics 49:231-4).

          Sequence analysis of CD28BP-15 indicated the amino acid sequence comprised a chimera derived principally from human, bovine, and rabbit sequences (Fig.

25 8B). The remaining clones selected from the libraries resulting from second round of recombination that displayed preferential binding to CD28 over CTLA-4 shared about 74 to about 99% sequence identity with CD28BP-15 based on sequence alignment comparisons (using DNASTAR or Vector NTI algorithm with default parameters as described above), illustrating the diversity of clones having the preferential binding properties. The nucleotide

30 and amino acid sequence identities of CD28BP-15 with human B7-1 were 73% and 61%, respectively (using DNASTAR or Vector NTI algorithm with default parameters). Based on amino acid sequence alignments, all of the selected CD28BP clones exhibiting preferential binding to CD28 over CTLA-4 relative to hB7-1 contained a substitution of

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valine for isoleucine at amino acid position 49 (Ile49Val) of the <u>mature</u> CD28BP-15 amino acid sequence (corresponding to alignment with the mature hB7-1 sequence). This substitution corresponds to a substitution at amino acid position 85 in the full-length CD28BP-15 and amino acid position 83 in full-length human B7-1, since CD28BP-15

5   includes two additional amino acid residues in the putative sequence. Although Ile49 does not appear to be directly involved in the interaction of B7-1 with CTLA-4 (see, e.g., Stamper, C. et al. (2001) *Nature* 410:608-611), substitution Ile49Ala was previously shown to completely abolish binding of B7-1 to both CD28 and CTLA-4, suggesting the importance of this residue in the ligand binding (Peach, R.J. et al. (1995) *J Biol Chem*

10   270:21181-7). Although the Ile49Val substitution is also considered a conservative replacement, our data suggest that mutations derived from naturally existing genes, in this case from the bovine B7-1 gene sequence, provide improved means to search for altered functional properties in proteins. Notably, bovine CD28 receptor is the only exception among CD28 and CTLA-4 receptor amino acid sequences analyzed from 12 different

15   species in which the hexapeptide MYPPPY and Gly-66 are not fully conserved in the receptor sequence (i.e., MYPPPY is replaced by LYPPPY, and Gly-66 is replaced by valine (see Metzler, W.J. et al. (1997) <u>Nat Struct Biol</u> 4:527-31). The hexapeptide MYPPPY is conserved in the F-G loop of both CD28 and CTLA-4 in a variety of mammalian species. Mutation of any residue in the MYPPPY sequence leads to reduced binding to B7-1 and

20   B7-2 (see <u>id.</u>), and all these residues, with the exception of Pro101, are in direct contact with B7-1 (Stamper, C. et al. (2001) <u>Nature</u> 410:608-611), suggesting that changes in this region of bovine CD28 receptor have driven the natural evolution of bovine B7-1 to acquire properties that also benefited the <u>in vitro</u> evolution of CD28BP described herein.

          CTLA-4BP 5x4-12c contained amino acid sequences corresponding

25   principally to the human, baboon, rhesus monkey and bovine B7-1 genes (Fig. 8A), and the nucleotide and amino acid sequences of CTLA-4BP were 97% and 96% identical with those of hB7-1, respectively. In addition, CTLA-4BP 5x4-12c contained three amino acid mutations that were not derived from any of the starting genes (Fig. 8A). The remaining clones with preferential binding to sCTLA-4-Ig over sCD28-Ig in the binding assays

30   exhibited 95-99% identity with CTLA-4BP 5x4-12c (e.g., using DNASTAR or Vector NTI algorithm with default parameters).

          Tyr-31 in the mature sequence of CTLA-4BP 5x4-12c (as measured from the N-terminus of the mature domain, using the amino acid position and numbering

corresponding to the amino acid sequence of hB7-1 (SEQ ID NO:278) was replaced by histidine (i.e., Try31His); the Tyr31His substitution was also present in a number of selected recombinant clones with preferential binding to CTLA-4, whereas all selected clones with preferential binding to CD28 retained the tyrosine at that position. Tyr-31 was also present at an equivalent position in all of the mature parental sequences. Tyr-31 of the mature CTLA-4BP and mature hB7-1 sequences (as measured from the N-terminus of the mature domain, using the amino acid position and numbering corresponding to the amino acid sequence of hB7-1) corresponds to Tyr-65 of the full-length CTLA4-BP and hB7-1 sequences, respectively (as measured from the N-terminus, which includes the signal peptide sequence). DNA sequence analysis of CTLA-4BP 5x4-12c showed a mutation in the codon corresponding to amino acid position 31 as measured from the N-terminus in mature hB7-1 – TAC – to codon CAC (at the corresponding position in mature form of CTLA-4BP 5x4-12c). Interestingly, it has been suggested that Tyr31 in human B7-2 may be replaced by phenylalanine without any apparent change in the ligand binding affinities (see, e.g., Freeman, G.J. et al. (1993) Science 262: 909-11); Azuma, M. et al. (1993) Nature 366:76-9), whereas a Tyr31Ala substitution in hB7-1 appears to completely abolish the binding of hB7-1 to both CD28 and CTLA-4 (see Peach, R.J. et al. (1995) J Biol Chem 270:21181-7). The present data demonstrate that the Tyr31His substitution, at least when present in the context of the shuffled CTLA-4BP sequence, does not significantly affect interaction with CTLA-4, whereas this mutation appears to contribute to the loss in binding to CD28, further supporting the suggestion that this residue plays an important role in the ligand binding of B7-1. Information regarding the three-dimensional structures of CD28BP and CTLA-4BP is useful in characterizing further the amino acid residues and structures that contribute to the preferential binding of NCSMs to their two respective ligands.

Sequence Round 2 CD28BP clones showed a range of amino acid and nucleotide diversity from wild-type B7-1 molecules. For example, the Round 2 CTLA4BP clones had a 93.8-96.9% amino acid identity with a WT B7-1 molecule and had a 96.2-97.7% identity at the nucleotide level.

The amino acid sequence of clone R2 CD28BP-15 differs from that of identical clones R2 CD28BP-3, -6, and -9 by only one amino acid residue at position 110; CD29BP-15 has a proline residue at position 110; clones CD28BP-3, -6, and -9 include a leucine residue at position 110.

The binding of labeled soluble ligand sCD28-Ig and sCTLA4-Ig to clones CD28BP-15 and CTLA-4BP 5x4-12c was further studied, as shown in Figures 9A-9H. Specifically, 293 cells were transiently (Figs. 9A-9B) or stably (Figs. 9C-9D) transfected with CD28BP-15 (solid circles) or hB7-1 (open squares), and with (dashed lines) and

5 without (solid lines) a FLAG-tag. 293 cells were stably transfected with CTLA-4BP 5x4-12c (solid triangles) or WT hB7-1 (open squares) (Figs. 9D-9E). Cells transiently (Figs. 9A-9B) or stably (Figs. 9C, 9D, 9E, 9F) transfected with a vector lacking a WT hB7-1, CD28BP or CTLA-4BP nucleic acid insert were used as negative controls (open diamonds). The transfectants were stained with increasing concentrations of labeled soluble CD28-Ig

10 (Figs. 9A, 9C, 9E) or soluble CTLA-4-Ig (Figs. 9B, 9D, 9F), prepared as described above, and the cells were analyzed by flow cytometry. Stable 293 transfectants expressing CTLA-4BP 5x4-12c (gray histograms), hB7-1 (gray histograms) and negative control transfectants (open histograms) were stained with anti-hB7-1 mAbs (Figs. 9G-9H), and the expression levels were analyzed by flow cytometry.

15 Staining of transfectants individually with sCD28-Ig or sCTLA-4-Ig indicated that CD28BP-15 transfectants bound sCD28-Ig at higher levels than transfectants expressing hB7-1 (as shown by increased MFI values), while sCTLA-4-Ig binding was significantly reduced, yet detectable (Figs. 9A-9D) (as indicated by reduced MFI). On the other hand, little or virtually no binding of sCD28-Ig to CTLA-4BP 5x4-12c transfectants

20 was detected even at high sCD28-Ig concentrations, while the same transfectants did bind sCTLA-4-Ig, although at somewhat lower levels than the transfectants expressing hB7-1 (Figs. 9E-9F) (as shown by MFI values). The lack of binding of sCD28-Ig to CTLA-4BP 5x4-12c was not due to the lack of expression, as was determined by the binding of anti-hB7-1 monoclonal antibody (mAb) to the transfectants (Figs. 9G-9H). In contrast to

25 CTLA-4BP 5x4-12c, mAbs specific for hB7-1 did not recognize CD28BP (data not shown).

To analyze whether increased expression of CD28BP-15 on the transfected cells contributed to the improved binding of sCD28-Ig, we fused a FLAG-tag (see according to Brizzard, B.L. et al. (1994) Biotechniques 16:730-5) to hB7-1 and the shuffled clones, and the binding of a FLAG-specific mAb M2 (see id.) to the corresponding

30 transfectants was studied. In seven separate transient transfections, the mean fluorescence intensity (MFI) of 293 cells transfected with CD28BP-FLAG and hB7-1-FLAG was 52±20 and 55±12, respectively (mean ± SEM). Although no significant difference in the expression of the FLAG-constructs was observed, significantly improved binding of CD28-

Ig was also observed using the FLAG-constructs (Figs. 9A-9B), strongly suggesting the improved binding of sCD28-Ig by CD28BP-15 was due to improved affinity rather than improved expression of CD28BP-15.

We also analyzed the ligand binding properties of all starting B7-1 genes by two-color analysis using sCD28-Ig and sCTLA-4-Ig to illustrate that CD28BP-14 and CTLA-4BP 5x4-12c showed evidence of truly new properties. 293 cells transfected with human, rhesus monkey, orangutan, and baboon B7-1 genes exhibited essentially identical binding profiles to SCD28-Ig and sCTLA-4-Ig, whereas feline B7-1 showed little or no binding to sCD28-Ig or sCTLA-4-Ig (data not shown). Bovine and rabbit B7-1 transfectants demonstrated sCD28-Ig binding in the same range as that observed for human and primate B7-1, but their level of binding to sCTLA-4-Ig was somewhat lower. However, the binding level of sCTLA-4-Ig to CD28BP-15 was consistently less than that of any of the other starting genes; the mean MFIs of sCTLA-4-Ig binding to human (n=4), bovine (n=4), rabbit B7-1 (n=2), and CD28BP-15 (n=2) were 247, 102, 205, and 30, respectively. Thus, CD28BP-15 and CTLA-4BP 5x4-12c displayed properties that were unique as compared to any of the starting genes.

B. Functional Assays.

To investigate the functional properties of shuffled R2 CD28BP molecules, nucleic acid sequences corresponding to representative clones R2 CD28BP-1 to R2 CD28BP-17 were transfected into B7-negative cell lines and the capacity of the resulting transfectants to activate human T cells was analyzed (using T cell proliferation assays with ^{3}H thymidine incorporation as described above). Representative results of selected clones are shown in Figure 10. When used to costimulate T cells in soluble anti-CD3 induced proliferation assays, these selected clones induced a range of proliferative responses from slight induction (potential antagonist) to improved induction over human B7-1 (Fig. 10). At one end of the range, clone CD28BP-8 (amino acid SEQ ID NO:59), which was shown to bind CD28, induced low T cell proliferation as compared to the wild-type hB7-1. CD28BP-8 induced a T cell proliferation level slightly greater than that induced by cells transfected with an empty vector (lacking a B7-1, CD28BP, or CTLA-4BP nucleic acid insert). In contrast, CD28BP-15 induced a T cell proliferation response significantly greater than that induced by WT hB7-1 (Fig. 10). Other CD28BP clones induced a T cell proliferation response about equal to that induced by WT hB7-1.

The enhanced co-stimulation of purified human T cells by clone CD28BP-15 was further investigated as follows (Figures 11A-11C). 293 cells were transiently (Fig. 11A) or stably (Fig. 11B) transfected with CD28BP-15 nucleic acid, hB7-1 nucleic acid, or an empty control vector lacking the B7-1 or CD28BP-15 nucleic acid insert, and the

5   irradiated transfectants were co-cultured with purified human T cells and anti-CD3 mAbs (to induce costimulation of T cells via the TCR) as described above. Mean ± SEM (standard error of mean) of counts per minute (C.P.M.) (^{3}H thymidine incorporation) obtained in three (Fig. 11A) or six (Fig. 11B) independent experiments are shown.

CD28BP-15 transfectants induced greatly increased proliferation of purified

10   human T cells cultured in the presence of anti-CD3 mAbs compared to cells transfected with hB7-1 (Fig. 11A). Moreover, we generated stable transfectants of CD28BP-15 and hB7-1 by selecting clones that expressed similar levels of the NCSM molecules based on binding of sCD28-Ig at saturating concentrations. Similar to transient transfectants, stable transfectants expressing CD28BP-15 induced a more potent T cell proliferation than those

15   transfectants expressing hB7-1 (Fig. 11B).

Irradiated stable transfectants expressing CD28BP-15 or hB7-1 and negative control cells transfected with a vector lacking the insert were co-cultured with purified human T cells, and the levels of IFN-gamma production were measured after a culture period of 48 hours. A representative experiment is shown in Fig. 11C; similar data were

20   obtained in three other experiments. Production of IFN-gamma in response to transfectants expressing CD28BP-15 was higher than that induced by hB7-1 transfectants (Fig. 11C). Approximately 10-fold fewer transient or stable transfectants expressing CD28BP-15 than those expressing hB7-1 were required to obtain a similar level of human T cell proliferation. Importantly, the maximum levels of T cell proliferation and IFN-gamma production were

25   also increased (Fig. 11C). We believe these results are likely attributable to the lack of negative signaling through CTLA-4. The increased affinity to CD28 and reduced affinity to CTLA-4 appears to have contributed to the CD28BP-15-mediated improved T cell response.

The effect of Round 2 CTLA-4BP clones on purified T cells in T cell

30   proliferation assays was investigated. In a representative T cell proliferation study performed as described above, 19 selected clones of the R2 library (designated as CTLA4BP-1 through CTLA4BP-19) having reduced binding to CD28, but relatively strong binding to CTLA-4, were found to reduce T cell proliferation in a soluble anti-CD3 induced

T cell proliferation assay (see Figure 12) compared to that induced by wild-type hB7-1. Cells transfected with an empty vector (lacking a B7-1 or CTLA-4BP nucleic acid insert), were used as the control. These selected CTLA-4BP clones produced a range of responses from reduced or suppressed induction of T cells relative to hB7-1 induction, to a significant

5   inhibition of T cell proliferation (Fig. 12).

The effect of exemplary clone CTLA-4BP 5x4-12c on purified T cells and on T cell proliferation and cytokine synthesis induced in MLR was further investigated. Representative results are shown in Figures 13A-13D. In the co-stimulation experiments, purified T cells were co-cultured in the presence of soluble anti-CD3 mAbs (5

10   micrograms/ml) and transient transfectants expressing hB7-1, CD28BP-15, CTLA-4BP 5x4-12c, or a vector control lacking the expressed sequence (Fig. 13A); a representative experiment is shown. Similar data were obtained in two other experiments. Increasing numbers of 293 cells stably transfected with CTLA-4BP 5x4-12c nucleic acid (solid triangles), hB7-1 nucleic acid (open squares), or a control vector lacking a B7-1, CTLA-

15   4BP, or CD28BP nucleic acid insert (open diamonds) were added to the MLR cultures, as shown in Fig. 13B. The data represent mean ± SEM of C.P.M. obtained in six separate MLR cultures, each performed using 4-6 replicate wells. MLR was cultured in the presence of 25000 irradiated 293 cells stably transfected with hB7-1, CTLA-4BP 5x4-12c or a control vector without an insert, as shown in Figs. 13C-13D. IFN-gamma and IL-10 levels

20   were measured by ELISA after an MLR culture period of 48 hours (Figs. 13C-13D). Six independent experiments were performed and the values obtained within one experiment are connected with a solid line. The production levels of IFN-gamma significantly increased ($P<0.05$) and those of IL-10 significantly decreased ($P<0.01$) for CTLA-4BP 5x4-12c compared to hB7-1 or vector control (paired Student's t-test).

25   Relative to CD28BP-15, hB7-1, and a control vector, CTLA-4BP 5x4-12c exhibited very little ability to co-stimulate human T cells cultured in the presence of soluble anti-CD3 mAbs (Fig. 13A). No co-stimulation of human T cells was induced by either transient or stable CTLA-4BP 5x4-12c transfectants, although efficient expression of the molecule on the surface of the transfectants was observed using anti-B7-1 mAbs (see Figs.

30   9G-9H, Fig. 13A, and data not shown). In fact, only 2 of 19 selected Round 2 clones (from Group I of the second round of recombination) that displayed preferential binding to CTLA-4 over CD28 had the capacity to induce a T cell response that was more than 10% of that

induced by hB7-1 (data not shown), illustrating the lack of sCD28-Ig binding to these clones correlated with the lack of signaling through CD28.

More importantly, CTLA-4BP 5x4-12c transfectants inhibited T cell proliferation induced in a mixed lymphocyte reaction (MLR) in a dose-dependent manner

5 (Fig. 13B). Moreover, CTLA-4BP 5x4-12c transfectants induced IL-10 production in MLR, but reduced IFN-g production, compared to hB7-1 or control transfectants (Figs. 13D and 13C, respectively), further supporting the notion that CTLA-4BP 5x4-12c has a dramatically altered biological function as compared to hB7-1. Several studies have suggested supporting roles for CTLA-4 (see, e.g., McAdam, A.J. et al. (1998) Immunol Rev

10 165:231-47; Waterhouse, P. et al. (1995) Science 270:985-8; Perez, V.L. et al. (1997) Immunity 6:411-7; Shrikant, P. et al. (1999) Immunity 11:483-93; van Elsas, A. et al. (1999) J Exp Med 190:355-66; Greenwald, R. et al. (2001) Immunity 14:145-155) and IL-10 (see, e.g., Groux, H. et al. (1997) Nature 389:737-42; Rizzo, L.V. et al. (1999) J Immunol 162:2613-22) in inducing and maintaining immunological tolerance. The results

15 of the present invention also suggest that CTLA-4BPs of the invention, including, e.g., CTLA-4BP 5x4-12c, are useful in downregulating the function of specific T cells in autoimmune diseases and the like, and are thus of benefit in therapeutic and prophylactic methods for treating such diseases.

Previous studies on B7 mutants showed that binding sites for CD28 and

20 CTLA-4 are largely overlapping and that mutations in B7 that affected the binding to one ligand (i.e., CD28 or CTLA-4) generally also affected the binding to the other ligand. However, only a limited number of variants were tested and they were generally designed based on information of expected ligand binding sites. For example, in contrast to the present results, mutations of human B7-1 (hB7-1) that were previously designed based on

25 structural information and predicted receptor binding sites generally produced equivalent effects on binding to CD28 and CTLA-4 (see, e.g., Peach, R.J. et al. J Biol Chem 270, 21181-7 (1995). The current data show that the frequency of functional variants with altered ligand binding properties is sufficiently high enough that a desired phenotype may be rapidly identified using the appropriate selection criteria and screening procedures.

30 Thus, appropriate screening of the sequences produced by recombination of known mammalian genomes serves as an effective means to identify those recombined sequences having novel functional properties without detailed knowledge of the receptor binding sites or the structures of the proteins. These results illustrate the advantages of the crossbreeding

of mammalian genes of different species to evolve proteins having novel functional properties. CD28BP and CTLA-4BP may also help in further deducing the mechanisms by which CD28 and CTLA-4 trigger positive and negative signals to T cells, respectively. The fact that B7-1 and B7-2 naturally bind to both CD28 and CTLA-4 has limited the potential

5   scope of their clinical applications. The chimeric CD28BP and CTLA-4BP molecules described herein have wide-ranging clinical applications; they are useful, for example, in the manipulation of T cell responses *in vitro* and *in vivo* in a variety of pharmaceutical and medical applications and vaccinations.

10   <u>EXAMPLE IV: Production of Soluble NCSM Polypeptides and Fusion Proteins and Nucleic Acids Encoding Them</u>

       The present invention also provides soluble NCSM polypeptides, and subsequences and fragments thereof that encompass a variety of formats, described herein, including, but not limited to, e.g., at least one of an extracellular domain (ECD), of a NCSM

15   polypeptide or a fragment, variant or homologue thereof, and an NCSM -ECD-Ig fusion protein or fragment, variant, or homologue thereof; and nucleotide and polynucleotide sequences encoding all such polypeptides, proteins, fragments, homologues, and variants.

       A soluble form of an NCSM polypeptide is useful in in vivo, ex vivo, or in vitro methods, including, e.g., therapeutic or prophylactic treatment or diagnostic methods,

20   administered as either a DNA plasmid expression vector (e.g., DNA vaccine; gene therapy applications) or protein, for treating or preventing various immunological disorders and diseases, including, e.g., cancers, including, but not limited to, e.g., colorectal cancer, breast cancer, pancreatic cancer, lung cancer, prostate cancer, naso-pharyngeal cancer, cancer, brain cancer, leukemia, melanoma, head- and neck cancer, stomach cancer, cervical cancer,

25   ovarian cancer, lymphomas, colon cancer, colorectal); allergy/asthma, autoimmune diseases, organ transplantation (e.g., graft versus host disease, and autoimmune diseases), chronic infectious diseases, including, but not limited to, e.g., viral infectious diseases, such as those associated with, but not limited to, e.g., hepatitis B virus (HBV), herpes simplex virus (HSV), hepatitis C virus (HCV), HIV, human papilloma virus (HPV), and the like,

30   and bacterial infectious diseases, such as, , but not limited to, e.g., Lyme disease, tuberculosis, and chlamydia infections; and other diseases described herein for non-soluble NCSMs of the invention, and as vaccine adjuvants in vaccine applications as described for

non-soluble NCSMS. Soluble NCSMs are also useful for diagnostic purposes, as for in vitro applications for testing and diagnosing such diseases.

In this example, selected CD28BP and CTLA-4BP clones were used for the preparation of corresponding soluble CD28BP and CTLA-4BP polypeptides and nucleic acids encoding such polypeptides. A CD28BP ECD polypeptide or fragment thereof may have a CD28/CTLA-4 binding affinity ratio about equal to, equal to or greater than the CD28/CTLA-4 binding affinity ratio of hB7-1, and other biological properties described below; a CTLA-4BP ECD polypeptide of fragment thereof may have a CD28/CTLA-4 binding affinity ratio equal to or greater than that of hB7-1, and other biological properties described below.

A.   Expression Vectors Encoding Soluble ECDs of NCSM Polypeptides

Mammalian expression plasmids encoding soluble (non-membrane bound) extracellular domains (sECDs) of NCSM polypeptides (or fragments thereof) were constructed by PCR amplification using pfu turbo polymerase (Stratagene, La Jolla, CA) of selected NCSM ECDs from plasmids containing a selected full-length NCSM DNA sequence. The PCR primers were designed to specifically anneal with the first or last 20-24 nucleotides of a particular nucleic acid region corresponding to a NCSM ECD (or fragment thereof), and flanked by restriction sites NheI and NotI, at their 5' and 3' ends, respectively. Amplicons of ~730 base pairs (bp) (specific base pair numbers for each clone are shown in Table 5) encoding individual ECDs (or fragments thereof) were digested with NheI and NotI (New England BioLabs, Beverly, MA), subsequently purified by low melt agarose gel electrophoresis (see procedure described in Sambrook, *supra*). A pcDNA3.1(+) expression plasmid (Invitrogen, Carlsbad, CA), which was used as the backbone vector, was digested with NotI and ApaI restriction enzymes and fused (ligated) in-frame to a nucleic acid sequence encoding a carboxy-terminal epitope fusion tag comprised of the E-epitope (Amersham Pharmacia Biotech) and hexa-His tag using standard cloning procedures. This vector fragment was then digested with NheI and NotI and to an ~5400 bp NheI-NotI fragment comprising the vector backbone and E-epitope/His-tag fusion sequence. This vector fragment was gel purified and ligated to each ~730 bp NheI-NotI NCSM ECD polynucleotide to produce soluble ECD NCSM expression plasmids. Mammalian expression plasmids encoding a soluble ECD form of wild-type human B7-1 were similarly prepared from plasmids containing the full-length WT human B7-1 DNA sequence.

Table 5

225

Clone ID	Nucleotide Position	Amino Acid Position	3' end ECD
wild-type huB7.1-ECD	1-726	1-242	PDN
CD28BP-8 ECD	1-735	1-245	IDQ
CD28BP-11 ECD	1-732	1-244	IDQ
CD28BP-15 ECD	1-735	1-245	IDQ
CTLA4-5X2-8C ECD	1-726	1-242	PDN
CTLA4-5X2-10C ECD	1-726	1-242	PDN
CTLA4-5X4-1F ECD	1-726	1-242	PDN
CTLA4-5X4-11D ECD	1-726	1-242	PDN
CTLA4-5X4-12C ECD	1-726	1-242	PDN
CTLA4-5X5-2E ECD	1-726	1-242	PDN
CTLA4-5X5-6E ECD	1-726	1-242	PDN
CTLA4-5X6-9D ECD	1-726	1-242	PDN
CTLA4-5X8-1F ECD	1-726	1-242	PDN

Clone ID	Nucleotide Position	Amino Acid Position	5' end Fc
P01857 Hu IgG1-Fc	298-690	100-230	PKSCDKTH...

Table 5 shows for positions of nucleotide residues and corresponding amino acid residues of the signal peptide sequence and representative ECD domains of selected CD28BP and CTLA-4BP clones, and equivalent positions in WT hB7-1 ECD, and the last

5 three amino acid residues at the 3' end of each ECD of a selected NCSM clone or WT hB7-1. The present invention provides for ECD domains of the NCSM polypeptides (and nucleic acid sequences encoding such polypeptides) that lack the signal peptide sequence, such that the first about 33 or about 34 amino acids (or about 99 or about 102 nucleic acids encoding same, respectively) of each NCSM ECD polypeptide (or nucleic acid encoding said polypeptide) are absent. For example, in CD28BP-15 polypeptide (SEQ ID NO:66),

10 the amino acid sequence of the signal peptide comprises MGHTMKWGSLPPKRPCLWLSQLLVLTGLFYFCSG (see Fig. 2A), and the nucleic acid sequence that encodes the signal peptide comprises at least about the first 102 nucleotide residues of SEQ ID NO:19. The signal peptide sequence of NCSM molecules may vary in

15 length. One of ordinary skill in the art can readily determine the amino acid sequence of an NCSM molecule that comprises the signal peptide sequence by aligning the full-length amino acid sequence of WT hB7-1 with the full-length amino acid sequence of the NCSM molecule, comparing the signal peptide sequence of WT hB7-1 with the corresponding sequence of the NCSM molecule, and determining the segment of the full-length amino acid

20 sequence of the NCSM molecule that corresponds to the signal peptide. The nucleic acid sequence that encodes the signal peptide of hB7-1 comprises the nucleotide residues of SEQ ID NO:273 that encode the signal peptide of SEQ ID NO:278. The nucleic acid sequence

that encodes the signal peptide of an NCSM molecule can be similarly determined by comparison of the full-length nucleic acid sequence of WT hB7-1 with the full-length nucleic acid sequence of the NCSM molecule.

Figure 14A shows a schematic representation of a hB7-1-ECD fused to an E-epitope amino acid sequence and a hexa-His tag amino acid. The amino acid sequences corresponding to the E-epitope and hexa-His tag, and selected amino acids of the ECD, are shown.

A representative plasmid expression vector, termed pNSCMsECD, comprising 6063 bps and encoding a soluble NCSM ECD, made by the above procedure is shown in Figure 15. The vector includes, among other things, selected elements of the pCDNA3.1(+) backbone, including an ampicillin resistant gene, A^R, and a bovine growth hormone (BGH) poly A termination sequence; nucleic acid sequence encoding an E-epitope/his tag; nucleic acid sequence encoding a NCSM-ECD; and a CMV promoter (e.g., known or WT CMV promoter, such as human CMV promoter, or recombinant or chimeric CMV promoter). A plasmid vector encoding a soluble hB7-1-ECD or fragment thereof can be made by substituting a hB7-1-ECD sequence or fragment thereof for the NCSM-ECD sequence shown in the figure.

A plasmid expression vector encoding either a soluble NCSM-truncated ECD (e.g., NCSM ECD fragment) or a soluble WT hB7-1-trunECD can be made using the procedure above by substituting a truncated NCSM ECD nucleic acid sequence or truncated hB7-1 ECD nucleotide sequence for NCSM ECD nucleotide sequence. Table 6 shows the positions of nucleotide residues and corresponding amino acid residues of the signal peptide sequence and exemplary truncated ECD domains of selected CD28BP and CTLA-4BP clones and WT hB7-1 ECD, and the last three amino acid residues at the 3' end of each truncated ECD of a NCSM clone or WT hB7-1.

Table 6

Clone ID	Nucleotide Position	Amino Acid Postion	3' end ECD
wild-type huB7.1-ECD	1-702	1-234	NTT
CD28BP-8 ECD	1-717	1-237	SKP
CD28BP-11 ECD	1-717	1-237	SKP
CD28BP-15 ECD	1-717	1-237	SKP
CTLA4-5X2-8C ECD	1-702	1-234	NTP
CTLA4-5X2-10C ECD	1-702	1-234	NTP
CTLA4-5X4-1F ECD	1-702	1-234	NTP
CTLA4-5X4-11D ECD	1-702	1-234	NTP
CTLA4-5X4-12C ECD	1-702	1-234	NTP

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Clone ID	Nucleotide Position	Amino Acid Position	5' end
CTLA4-5X5-2E ECD	1-702	1-234	NTP
CTLA4-5X5-6E ECD	1-702	1-234	NTT
CTLA4-5X6-9D ECD	1-702	1-234	NTP
CTLA4-5X8-1F ECD	1-702	1-234	NTP

Clone ID	Nucleotide Position	Amino Acid Position	5' end Fc
X70421 Hu IgG1-Fc	85-768	29-256	DKTH...

The invention also provides for ECD domains of the NCSM polypeptides (and nucleic acid sequences encoding such polypeptides) that lack the signal peptide sequence; the first 34 amino acids (or 102 nucleic acids encoding same) of each NCSM

5 ECD polypeptide (or nucleic acid encoding a NCSM ECD polypeptide) are absent. In secreted forms, the signal sequence is cleaved.

B. Expression and Purification of Soluble ECD and NSCM Polypeptides.

Soluble polypeptides from the CTLA-4BP-ECD, CD28BP-ECD, and hB7-1-ECD expression vectors described above was expressed by transfection of these vectors into

10 cells of a human HEK 293 cell line using Superfect Reagent (Qiagen) and expression of the sequence encoding the NCSM ECD or a fragment thereof. In each case, soluble protein was purified from crude culture supernatants using a Hi-Trap anti-e-epitope mAb affinity column (Amersham-Pharmacia, Piscataway, NJ) followed by buffer exchange into PBS, according to the manufacturer's instructions. Purity of a recovered fusion protein was

15 assessed by SDS-PAGE followed by either coumassie stain or immunoblotting with a mouse anti-penta-His mAb (Serotech, UK), performed according to manufacturer's instructions and using known methods as described in, e.g., Rapley and Walker; Harlow and Lane; Colligan; Sambrook, all *supra* (data not shown). The SDS-PAGE results for soluble hB7-1-ECD and soluble CD28BP-15 sECD revealed a molecular weight (MW) of ~50 kDa

20 for each, as shown in Figure 16. A reference mixture spotted in the far-left lane indicated bands of compounds of known MWs of 188 kiloDaltons (kDa), 98kDa, 56 kDa, and 31kDa, respectively, for comparison. SDS-PAGE analysis showed a hB7-1-Ig fusion protein homodimer of ~140 kDa; this dimer is believed to contain a covalent linkage between cysteine residues of the hinge-CH2-CH3 domain of the Fc. A hB7-1-Ig monomer thus has

25 an apparent MW of ~70 kDa. A ~70-kDa monomer of hB7-1-Ig-delta Cys mutant fusion protein was also observed (see Figure 16). It is believed the deleted cysteine (δCys) mutant prevents covalent dimerization of two individual B7-1-ECD/hinge-CH2-CH3 (Ig) molecules.

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C.   Expression Vectors Encoding Soluble NCSM-Ig Fusion Proteins.

Mammalian expression plasmids encoding a soluble NCSM-ECD-Ig fusion protein and soluble WT hB7-1-ECD-Ig fusion protein were constructed first by PCR amplification using pfu turbo polymerase (Stratagene, La Jolla, CA) of selected NCSM

5   ECDs or hB7-1-ECD from plasmids containing a full-length NCSM DNA sequence and hB7-1 DNA sequence as described above. The PCR primers were designed to anneal specifically with the first or last 20-24 nucleotides of a particular nucleic acid region corresponding to the ECD of a specific NCSM or hB7-1, and flanked by restriction sites BamHI and BsteII, at their 5' and 3' ends, respectively. In some instances, the small peptide

10   linker forming the in-frame translational coupling between the NCSM ECD (or hB7-1) and the IgG1 Fc contained the sequences valine-threonine (VT) or glycine-valine-threonine (GVT), depending upon the nucleotide sequence compatibility of the 3' codon of the NCSM ECD. Incorporation of the BsteII restriction site at the fusion junction creates the in-frame valine-threonine linker. The factor Xa cleavage site (IEGR) was inserted between the 3'

15   end of the NCSM ECD (or hB7-1 ECD) and 5' end of the GVT or VT linker to allow production of sECD void of the Fc domain (Figure 14B).

DNA sequences encoding human IgG1 Fc were obtained from human spleen mRNA (Clontech, Palo Alto, CA) using a RT-PCR kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, and primers specific to the first or last 20-28 nucleotides

20   of the sequence corresponding to the entire human IgG1 Fc hinge domain or a fragment thereof (see, e.g., the protein sequences shown at GenBank Accession Nos. P01857 and X70421, respectively; other Fc sequences can also be used) flanked by restriction sites BstEII and EcoRI, at their 5' and 3' ends, respectively (Figure 14B). Alternatively, a variant derived from a human IgG1 Fc hinge domain (e.g., GenBank Access. No. P01857 or

25   X70421) can be prepared that imparts specific desirable biological and pharmacological properties to the soluble NCSMs.

The IgG1-Fc amplicon (~730 bp) was digested with BstEII and EcoRI and subsequently cloned into pCDNA3.1(+) expression vector (serving as a backbone vector) digested with BamHI and EcoRI. The NCSM-Ig expression plasmids were constructed by

30   ligating (fusing in-frame) low melt agarose purified NCSM BamHI-BstEII DNA fragments (~730 bp), Ig-Fc BstEII-EcoRI DNA fragments (~730) and pCDNA3.1(+) BamHI-EcoRI DNA fragment (~5400 bp) to produce soluble NCSM-ECD-IgFc fusion expression vectors (see Figure 17).

A sequence complementary to either that corresponding to the sequence shown at GenBank Accession No. X70421 or P01857 and an IgG Fc variant containing two amino acid substitutions (D234E and L241M) were cloned and deduced by DNA sequence analysis using known methods. The NCSM-Ig fusions proteins may contain an IgG Fc

5   sequence corresponding to that shown at GenBank Accession No. X70421 or P01857 or IgG Fc variant sequence as the fusion partner.

Figure 14B shows a representation of a soluble WT human B7-1-ECD-Ig sequence, including the signal domain, ECD, Factor Xa (IEGR), VT or GVT linker, and human B7 hinge CH2-CH3 domain of the Fc region of IgG1 corresponding to the sequence

10   shown at GenBank Accession No. P01857. The amino acid residues positioned at the beginning and end of a representative ECD domain and 5' end of the human B7 hinge CH2-CH3 domain are shown. A NCSM-ECD-Ig sequence would be comparable to that shown for hB7-1ECD-Ig in Figure 14B.

Nucleotide sequences encoding truncated NCSM ECDs or truncated hB7-

15   1ECDs were also used to make NCSM-trunECD-Ig and hB7-1-trun ECD-Ig expression constructs and fusion proteins, respectively. Truncated NCSM ECDs typically contained at least one less amino acid residue than the full-length NCSM ECD; nucleotide sequences encoding truncated NCSM ECDs comprised corresponding fewer nucleotides.

The nucleotide positions and corresponding amino acid positions of an

20   exemplary full-length ECD or truncated ECD of selected NCSM clones and hB7-1 used for construction of expression plasmids encoding the fusion proteins are shown in Tables 5 and 6. Table 5 shows the nucleotide positions and corresponding amino acid positions of the hIgG1 Fc sequence shown at GenBank Accession No. P01857, and amino acid residues at the 5' end of this Fc region. Table 6 shows the nucleotide positions and corresponding

25   amino acid positions of the hIgG1 Fc sequence at GenBank Accession No. X70421, and amino acid residues at the 5' end of this Fc region. The invention also provides for fusion proteins in which the ECD domains of the NCSM polypeptides (and nucleic acid sequences encoding such polypeptides) lack the signal peptide sequence; in this aspect, the first 34 amino acids (or 102 nucleic acids encoding same) of each NCSM ECD polypeptide (or

30   nucleic acid encoding NCSM ECD polypeptide) is absent. In secreted forms, the signal sequence is cleaved.

A representative plasmid vector encoding a soluble hB7-1-ECD-Ig fusion protein, phB7-1-ECD-Ig, is shown in Figure 17. The vector includes, among other things,

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selected elements of the pCDNA3.1(+) backbone, including an ampicillin resistant gene, A^R, and a bovine growth hormone (BGH) poly A termination sequence; nucleic acid sequences encoding a hB7-1-ECD/IgG1 Fc fusion protein; and a CMV promoter (e.g., known or WT CMV promoter, such as human CMV promoter, or recombinant or chimeric

5   CMV promoter). A plasmid vector encoding a soluble NCSM-ECD-IgG1 fusion protein or fragment thereof can be made by substituting a NCSM-ECD sequence or fragment thereof for the hB7-1 ECD sequence in the vector shown in Figure 17. Plasmid vectors encoding a hB7-1-trunECD-Ig or NCSM trunECD-Ig fusion protein are also be made using the same procedure.

10   In another aspect, a non-dimerizing Ig-Fc domain (PKSCDKTHTCPPCP → PKSSDKTHTSPPSP) was engineered by PCR mutagenesis (Stratagene, La Jolla, CA) to mutate the cysteine residues within the Ab hinge region to serine residues so as to prevent the formation of NCSM-ECD-Ig or hB7-1-ECD-Ig homodimers covalently linked by disulfide bonds between the hinge-CH2 cysteines of neighboring NCSM-ECD-Ig or hB7-1-

15   ECD-Ig molecules. This non-dimerizing Ig-Fc domain can alternatively be used as the Ig portion in an NCSM-ECD-Ig or hB7-1-ECD-Ig fusion protein prepared as described above. Affinity purified huB7-1-ECD-IgδCys, comprising hB7-1-ECD fused to Ig in which the cysteines were mutated (represented by delta or δCys) was shown to have a molecular weight of ~70 kDa (molecular size of non-disulfide linked Fc fusion monomer) (Figure 16).

20   The present invention provides similarly prepared Cys-mutant Ig fusion proteins, NCSM-ECD-IgδCys or NCSM-trunECD-IgδCys, and nucleic acid sequences encoding such proteins.

      D.   Expression and Purification of Soluble NCSM ECD-Ig Protein

               Soluble protein from CTLA-4BP-ECD-Ig fusion and CD28BP-ECD-Ig

25   fusion expression vectors was expressed by transfection into cells of a human HEK 293 cell line using Superfect Reagent (Qiagen) and purified from crude cultured supernatants using a Hi-Trap Protein-A affinity column (Amersham-Pharmacia, Piscataway, NJ) followed by buffer exchange into PBS, according to the manufacturer's instructions. Purity of the recovered fusion protein was assessed by SDS-PAGE followed by either silver stain or

30   immunoblotting with a goat anti-human IgG Fc specific horseradish peroxidase (HRP) mAb (Kirkegaard and Perry Laboratories), according to manufacturer's instructions and known methods as described in, e.g., Rapley and Walker, Sambrook, and Colligan, all *supra.*

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As an example, a soluble wild-type human B7-1-ECD-Ig fusion protein (e.g., comprising, in part, an ECD fragment, "truncated ECD," or full-length ECD of hB7-1) was purified from human HEK 293 cells transfected with the expression plasmid, phuB7-1 ECD-Ig, using Protein-A affinity chromatography and fractionated on a SDS-PAGE. Serial

5 dilutions of soluble human B7-1 ECD-Ig fusion protein showed a band which co-migrated with a commercially available form of soluble WT human B7-1-ECD-Ig or B7-2-ECD-Ig fusion protein from R&D Systems, thus demonstrating that a protein of the correct molecular weight for a soluble WT hB7-1-ECD-Ig fusion protein was produced in vitro (data not shown). The results indicated that WT hB7-1 ECD-Ig fusion protein is

10 predominantly an ECD-Ig homodimer fusion protein. (Soluble hWT hB7-1 ECD monomer was shown to have a molecular weight on SDS-PAGE of about ~50 kDa.) SDS-PAGE analysis revealed the affinity purified CD28BP-15 ECD-Ig and CTLA-4BP 5X4-12C ECD-Ig fusion proteins co-migrated with WT human B7-1 ECD-Ig fusion proteins, as shown in Figure 18, and thus have molecular weights nearly identical to or at least approaching that

15 of WT hB7-1 ECD-Ig fusion protein. A reference mixture included at the far-left shows bands of compounds of known MWs (Figure 18).

Crude supernatants from HEK 293 cells transfected with expression plasmids encoding either a soluble (e.g., truncated or full-length ECD) fusion protein form of various CTLA-4BPs and CD28BPs of the invention, a WT hB7-1-ECD-Ig, or a pCDNA3.1(+)

20 vector control were fractionated on an SDS-PAGE, blotted to nitrocellulose and hybridized with a goat anti-human IgG Fc specific HRP mAb using known Western blotting methods (e.g., Rapley and Walker; Sambrook; Harlow and Lane, all *supra*). Results, shown in Figure 19, showed a predominant band and fainter band around ~140 kD and ~70 kDa, respectively, which correspond to the predicted molecular weight of dimeric and

25 monomeric forms of the NCSM fusion proteins. 8 potential N-glycosylation sites located in the ECD. Note that the MWs are approximate weights, since the proteins may be glycosylated. A band co-migrating with soluble WT hB7-1ECD-Ig was visible for all CTLA-4BP ECD-Ig and CD28BP ECD-Ig fusions. NCSM-trunECD-Igs also showed similar results to hB7-1-trunECD-Igs. The supernatant from the negative control

30 transfection (HEK 293 cells transfected with a pCDNA3.1(+) vector) did not produce a detectable band. Oligomeric or multimeric forms (NCSM-ECD-Ig dimers, trimers, etc.) were observed for CTLA-4BPs (Clones 5X4-11D, 5X4-12C, 5X5-2E, 5X8-1F) and CD28BPs (Clones 8 and 11).

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E.   Construction of Stable Cell Lines Expressing Soluble NCSM-ECD and
      NCSM-ECD-Ig

Stable 293 cell lines expressing soluble NCSM-ECD (NCSM-sECD)
polypeptides or soluble NCSM-ECD–Ig fusion proteins were produced by electroporating

5   HEK 293 cells with DraIII digested expression plasmids comprising nucleic acid sequences
encoding such polypeptides or proteins according to the known methods as described in,
e.g., Ausubel and Sambrook, both *supra*. Stable integrants were selected using DMEM
containing 10% FCS and 2mg/ml G418 antibiotic (Geneticin, Gibco-BRL). Purification
was performed as described above except the eluate containing NCSM-Ig fusions from the

10   Protein-A column was further purified by standard gel-filtration chromatography (size-
exclusion chromatography) (see, e.g., Rapley and Walker; Sambrook, both *supra*) using a
Superdex 200 10/30 (24 ml) column (Amersham-Pharmacia) (following manufacturer's
instructions) to remove non-NCSM proteins. The approximate apparent molecular weights
(App MW) of purified soluble NCSMs as determined by this gel-filtration analysis are

15   shown in Table 7 below.

Table 7

Molecule Type	App MW (kDa)
sCD28BP-15-ECD	~49.04
HuB7-1/IgFc (commercial, R&D Systems)	~643.39
wtHuB7-1-Ig	~320.02
wtHuB7-1-IgδCys	~403.31
CTLA-4BP 5X4-12C-ECD-Ig	~330.28
CTLA-4BP 5x4-12C-ECD	~106
CD28BP-15-ECD-Ig	~345.54

25   In addition to monomers of NCSM-ECD-Ig and NCSM-trun-ECD-Ig, the
present invention includes aggregates and multimers (e.g., crosslinked forms) of the soluble
NCSM polypeptides of the invention, such as, e.g., NCSM-ECD-Ig and NCSM-trun-ECD-
Ig, where the Ig portion comprises an Fc region or variant thereof as described above.

F.   In Vitro Characterization of Biological Activities.

30   1.   T Cell Proliferation Assays Using Soluble NCSM Fusion Proteins
                 Soluble NCSM were generated and purified as described above. Human
wild-type B7-1 was also expressed using the same methods. In addition, a commercial
human wild-type B7-1-Ig fusion protein was obtained from R&D Systems (see also Table
7). To characterize the biological properties of these molecules, two different formats were

35   used to further analyze the effects of crosslinking and non-crosslinking on the function of
the molecules. More specifically, we analyzed the fusion proteins both as standard non-

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crosslinked soluble molecules as well after preincubation with mAbs specific for the Fc

portion of human IgG (crosslinked molecules). Crosslinking has previously been shown to

affect the function of wild-type human B7-1 (Rennert et al., Intl Immunol. 1997

Jun;9(6):805-13). Crosslinked molecules comprise at least two molecules of interest, e.g.,

5   multimers, such as two NCSM molecules. A non-crosslinked NCSM molecule comprises

one NCSM molecule.

Purified human T cells were used in these studies. T lymphocytes were

sorted using a Moflow flow cytometer by the methods described previously in "T Cell

Proliferation Assay" in the "Materials and Methods" section above and used at 1×10^5

10   cells/well in U-shaped 96-well assay plate. T cells were cultured in the presence of soluble

anti-CD3 mAbs (Pharmingen, San Diego, CA) to deliver a primary signal (also called

Signal 7). The secondary signal to T cells was delivered by adding the purified Ig fusion

proteins; e.g., WT hB7-1-ECD-Ig, CD28BP-ECD-Ig, or CTLA-4BP-ECD-Ig were added at

various concentrations, and anti-CD28 mAbs (Pharmingen) were used as a positive control.

15   To obtain crosslinked Ig-fusion molecules, purified Ig fusion proteins were pre-incubated

with 5-fold excess of affinity-purified goat anti-human IgG Fc portion (KPL, Gaithersburg,

MD) for 30 minutes (min) on ice prior to use. The cross-linked complex was then added

into 96-well plate containing T cells in total volume of 200 ul of Yssel's medium (Yssel et

al. (1984) J Immunol Methods 72(1):219) supplemented with 10%FBS. Assay plates were

20   incubated for total of 3 days. The cultures were pulsed with 1microCi/well of ^{3}H-thymidine

(Amersham, Piscataway, NJ) during the last 8 hours of incubation and then harvested. ^{3}H-

thymidine incorporation (cpm) was calculated from triplicate cultures.

A representative experiment using crosslinked fusion proteins and

purified human T cells is shown in Figure 20A. Increasing concentrations (conc) of soluble

25   Ig-fusion proteins of hB7.1 (solid square), CD28BP-15 (open triangle) and a control

antibody human IgG (open circle) were added at various concentrations to the cultures as

indicated. A fixed concentration (125 micrograms/milliliter (25 ug/ml)) of goat anti-human

IgG Fc was preincubated with soluble Ig-fusion proteins prior to use. The data represent a

mean+/- SD of C.P.M. Both crosslinked CD28BP-15-ECD-Ig-fusion protein and WT hB7-

30   1-ECD-Ig fusion protein induced a strong proliferative effect on purified human T cells

cultured in the presence of anti-CD3 mAbs. CD28BP-15-ECD-Ig fusion protein induced a

more significant T cell proliferation response than did hB7-1-ECD-Ig fusion protein after

cross-linking with goat anti-human IgG Fc antibodies. Figure 20E represents a summary of

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data from 4 separate experiments using crosslinked fusion proteins comprising NCSM multimers and purified human T cells as described in Figure 20A above. The data represent a mean +/- SEM of C.P.M. Based on these analyses using anti-human IgG mAbs to make multimeric, crosslinked NCSM molecules, crosslinked CD28BP-ECD-Igs (e.g., CD28BP-

5   15-ECD-Ig) promoted activation of the CD28 receptor to a greater extent than did crosslinked WT hB7-1-ECD-Ig.

These data indicate that the formulation/multimerization of the soluble NCSM fusion proteins and soluble NCSMs significantly affects their biological properties. That is, soluble multimeric forms of NCSM molecules (e.g., comprising two or more

10   subunits of a NCSM-ECD-Ig, NCSM-trunECD-Ig, NCSM-ECD, NCSM-trunECD or other protein and fusion protein variants thereof) have biological properties that are different from corresponding soluble monomeric forms. In such multimeric forms, each NCSM subunit of the multimer need not be identical. Soluble multimeric forms comprising two or more NCSM subunits (e.g., NCSN-ECD subunits) can be made by crosslinking, using leucine

15   zippers, or engineering the subunits by other means known to those skilled in the art for making multimers or aggregates. When tested as soluble molecules without crosslinking, soluble monomeric CD28BP-15-ECD-Ig fusion proteins inhibited PHA-induced proliferation of human PBMC (see below). However, similar to the membrane-bound version of CD28BP-15 (full length), the crosslinked soluble CD28BP-ECD-Ig molecule

20   strongly enhanced the proliferation of purified T cells in the presence of soluble anti-CD3 mAbs. These data indicate that crosslinked or aggregated (e.g., multimeric) forms of soluble forms of the NCSM polypeptides (e.g., soluble NCSM-ECD-Ig, NCSM-trunECD-Ig, NCSM-ECD, NCSM-trunECD and other protein and fusion protein variants thereof) are promising drugs to activate the immune system, which is expected to be beneficial in the

25   treatment of malignant diseases (e.g., cancer), infectious diseases, and immunodeficiencies. The crosslinking can be generated in vitro (e.g., as described in assays above) or in vivo (e.g., through high-affinity binding to Fc receptors on antigen-presenting cells). In addition, when using cell-based vaccines, the crosslinking can be caused by transfecting receptors for human IgG (Fc receptors) into the cells that are used as vaccines (and the NCSM-Ig fusion

30   binds to the Fc receptors expressed on the cells that are used as vaccines. In contrast, without prior crosslinking, the soluble NCSM polypeptides inhibited the PHA-induced proliferation of human PBMC, indicating that they have inhibitory effects on human T cell function. Furthermore, soluble CD28BP-ECD (without Ig portion) strongly inhibited T cell

proliferation in vitro. Therefore, these soluble NCSM polypeptides are promising drugs for the treatment of autoimmune and inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, and organ transplantation.

When PBMCs were cultured in the presence of the soluble molecules of

5   CD28BP-15-ECD-Ig or CTLA-4 BP 5x4-12c-ECD-Ig without prior crosslinking (and no PHA), no T cell activation was observed except in the case of insect cell derived commercial wild-type B7-1-Ig (see Table 7). An increasing concentration of non-crosslinked ECD-Ig fusion proteins of hB7.1 (solid square), and a control antibody human IgG (open circle), and commercially obtained insect cell derived human B7-1-Ig fusion

10   proteins (R&D Systems) (solid triangle), were added to cultures of PBMC and proliferation was measured as described above (Figure 20B). The insect cell derived human B7-1-Ig fusion proteins induced proliferation of human PBMC, whereas the hB7-1 expressed in 293 cells did not induce T cell proliferation. When these proteins were analyzed by gel filtration, it was evident that the protein expressed in insect cell had a 2-fold higher

15   molecular weight than hB7-1-Ig fusion expressed in 293 cells (Table 7). These data further support the conclusion that higher molecular weight aggregates (e.g., crosslinked or multimeric molecules) improve the capacity of these soluble NCSM polypeptides to signal through their respective ligands.

We also performed a PHA-activated T cell proliferation assay. In this assay,

20   PBMCs were isolated from human blood by centrifugation over Histopaque-1077. PBMCs were used at 1×10^5 cells/well in 96-well round bottom plate (Costar); each well contained a total volume of 200 ul Yssel's medium (Yssel et al. (1984) J Immunol Methods 72(1):219) supplemented with 10% FBS. 1ug/ml of PHA (Phytohemagglutinin) (Sigma, St. Louis MO) was added into these cultures. PHA is a plant protein (lectin-like molecule) that is

25   extracted from Phaseolus vulgaris (red kidney bean) and binds to various sugars and sugar residues in oligosaccharides. PHA is used as a T cell mitogen to activate T cells (including, e.g., memory T cells, naïve T cells, etc.). Increasing concentrations (ug/ml) of soluble non-crosslinked CD28BP-15-ECD-Ig (open triangle), non-crosslinked hB7-1-ECD-Ig (solid square), R&DhB7-1-Ig fusion proteins (R&D Systems, Minneapolis, MN) (solid triangle),

30   made as described previously, and CTLA-4-Ig ligand (R&D Systems) (solid diamond) and control hIgG antibody (open circle)(Jackson ImmunoResearch Lab. Inc., West Grove, Penn.) were added to these cultures as shown in Figure 20C. The data represent mean +/- SEM of a representative of 4 experiments, each preformed using triplicate wells. When the

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soluble CD28BP-15-ECD-Ig fusion proteins were cultured as soluble molecules without prior crosslinking in the presence of PHA, they inhibited PHA-induced proliferation of human PBMC in a dose-dependent manner (Figure 20C) and thus are useful in immunosuppressive applications. Wild-type hB7-1-ECD-Ig did not affect PHA-induced proliferation of human PBMC under the same culture conditions (Fig. 20C). At most concentrations, CTLA-4-Ig inhibited PHA-induced proliferation of human PBMC more than did hB7-1-ECD-Ig.

Furthermore, we studied the effect of soluble CD28BP-15-ECD (without Ig-fusion) on PHA-activated PBMC. As shown in Figure 20D, soluble CD28BP-ECD inhibited proliferation in a dose-dependent manner and the inhibition was greater than that induced by soluble hB7-1-ECD. These data are in line with the conclusion that soluble CD28BP-15-ECD, in contrast to crosslinked soluble CD28BP-15-ECD-Ig, acts as an antagonist of CD28 signaling. In other words, the level of crosslinking and multimerization determines whether soluble NCSM induce a positive signal through their receptors CD28 and CTLA-4, or whether they act as antagonists by binding to the receptors without significantly inducing activation of the receptor (and thereby preventing the interaction of the endogenous ligands with these receptors). The effect of soluble non-crosslinked CD28BP-15-ECD-Ig fusion protein and CD28BP-15-ECD on mixed lymphocyte reaction (MLR), as described above, was studied using populations of PBMCs from two human donors. PBMCs from one donor acted as a responder; PBMCs from the second donor, which were irradiated at 2500 rads, served as a stimulator for the responder. Increasing concentrations (ug/ml) of soluble non-crosslinked CD28BP-15-ECD-Ig (open triangle), non-crosslinked hB7-1-ECD-Ig (solid square), non-crosslinked CD28BP-15-ECD (solid triangle), non-crosslinked hB7-1-ECD (open square), and negative control hIgG antibody (open circle) were added to these cultures as shown in Figure 20F. These data represent an average of counts per minute (CPM) from 4 MLR experiments. CD28BP-15-ECD-Ig and CD28BP-ECD inhibited mixed lymphocyte reaction to a greater extent than did hB7-1-ECD-Ig and hB7-1-ECD.

G.   Multimeric NCSM Molecules Using Leucine Zippers

Multimeric NCSM molecules are generated using leucine zippers. In this approach, leucine repeats are utilized to generate multimeric NCSM molecules that have properties which are equivalent or substantially identical to properties of the crosslinked NCSM multimers described above generated by crosslinking using goat anti-human IgG Fc

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mAbs (and crosslinked NCSM fragments having such properties). The use of leucine
zippers to oligomerize proteins is well known (see, e.g., Rieker and Hu (2000) Methods
Enzymol. 323:282-96; Behncken et al. (2000) J. Biol. Chem. 275:17000-17007; Su et al.
(1999) J. Immunol. 162:5924-5930). Leucine zippers comprise a motif comprising parallel

5   α-helical coiled coils as exemplified by those found in transcription factors GCN4
(RMKQLEDKVEELLSKNYHLENECARLKKLVGER), Fos
(LTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAA), and Jun
(RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMN). The motif is
represented by the occurrence of leucines in every seventh position and hydrophobic and

10   branched amino acids occupying position 1 over four or five heptad repeats. These motifs
act as molecular clamps and facilitate homo- and hetero- dimer formation of proteins.
Homodimerization or higher order self-oligomerization of a protein is also accomplished
using other zipper motifs (e.g., Zhang et al. (1999) Current Biol. 9:417-420) or mutated
peptide sequences derived from transcription factors, GCN4, Fos, or Jun.

15   Soluble monomeric NCSM molecules are modified with leucine zippers
using standard molecular biology methods utilizing either natural (genomic
eukaryotic/prokaryotic DNA or plasmid/viral DNA) or synthetic DNA encoding known or
newly discovered oligomerization motifs. These sequence motifs are engineered as
molecular zipper cassettes containing unique restriction enzyme sites to make in-frame

20   fusion with either the N-terminus or C-terminus of a soluble monomeric NCSM. For
example, a DNA sequence encoding the leucine zipper comprising the restriction site Not I
at the 5' end and restriction site Apa I at the 3' end is used to facilitate cloning into a vector
backbone, such as, e.g., pCDNA3.1 (GCGGCCGCa<GCN4>TAGGGGCCC; GCN4
nucleotide sequence can be codon optimized to improve translation in a particular cell of

25   interest, such as a human cell). This allows for easy shuttling of DNA fragments encoding
soluble monomeric NCSM-ECD polypeptides or monomeric NCSM-ECD Ig-fusion
polypeptides to oligomerization expression vectors. These expression vectors can also
include an E-epitope and hexa-His tag for diagnostic and purification of soluble proteins.

         H.   Variations of Soluble NCSM-ECD-Ig Fusion Proteins and Related
30   Nucleic Acid Sequences
               Any of the NCSM polypeptide homologues of the invention are optionally
utilized in the construction of Ig fusion proteins and nucleic acids encoding them. Full-
length NSCM polypeptides of the invention can be used. Furthermore, various fragments of

each NCSM polypeptide can be utilized in the construction of fusion proteins, including, e.g., the entire ECD of a NCSM polypeptide (such as CD28BP-15 or CTLA-4BP 5x4-12c); various lengths or subsequences (e.g., truncated regions or fragments) of the ECD of a NCSM polypeptide; the cytoplasmic region of a NCSM polypeptide (and truncated regions and subsequences or fragments thereof); the transmembrane domain region of a NCSM (and truncated regions and subsequences or fragments thereof), etc. Various additional sequences can also be added to the NCSM-Ig fusion proteins, e.g., various linker sequences (such as, e.g., Val-Thr), various proteolytic cleavage sites (such as, e.g., Factor Xa cleavage sites (IEGR), subtilisin, etc.), various Ig domains (or portions thereof), markers, purification sequences, restriction enzyme cleavage sites, and the like. As noted throughout, non-Ig sequences can also be fused to the given NCSM sequences to produce fusion proteins.

For example, as illustrated above, NCSM polypeptide sequences of the invention were utilized to construct Ig fusion proteins incorporating both linkers (V-T and G-V-T) and Factor Xa Cleavage sites. See, e.g., Figures 14A-14B, Tables 5-6. The NCSM portions of these fusion proteins were longer than the truncated NCSM sequences used to construct the fusion proteins as described elsewhere herein. Various sequence lengths of NCSMs (both amino acid and nucleotide) can be utilized in constructing Ig fusions as well as myriad, e.g., linkers and other sequences, etc. Various configurations of linkers, NCSM lengths, etc. are all aspects of the present invention. Any of the NCSM sequences described herein can be fused using essentially the same strategy.

The invention also provides nucleic acids encoding any of the variant soluble NSMC polypeptides and fusion proteins described above or fragments thereof. Also included are vectors and expression cassettes including such nucleic acids.

EXAMPLE V: Construction of an Expression Cassette

A.   Construction of Vector pMaxVax10.1.

This example describes the construction of an exemplary vector for expression in mammalian cells. The mammalian expression vector pMaxVax10.1 (see Figure 21) comprises, among other things: (1) a promoter for driving the expression of a transgene in mammalian cells;(2) a polylinker for cloning of one or more transgenes; (3) a polyadenylation signal (polyA); and (4) a prokaryotic replication origin and antibiotic resistant gene for amplification in E. coli.

1.   Construction of minimal plasmid for amplification in E. coli.

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The minimal plasmid Col/Kana comprises the replication origin ColE1 and the kanamycin resistant gene (Kana^r). The ColE1 replication origin mediates high copy number plasmid amplification. Alternatively, low copy number replication origins, such as p15A (from plasmid pACYC177, New England Biolabs Inc.) can be used.

5   The ColE1 origin was isolated by polymerase chain reaction (PCR) methods known in the art from vector pUC19 (New England Biolabs Inc.). To link the ColE1 origin to the Kana^r gene, NgoMIV (or "NgoMI") and DraIII recognition sequences where added to the 5' and 3' PCR primers, respectively. NgoMIV and DraIII are unique cloning sites in the pMaxVax vector. For subsequent cloning of the mammalian transcription unit the

10   5'forward primer contains the additional restriction site NheI downstream of the NgoMIV site and the 3'reverse primer additional EcoRV and BsrGI cloning sites upstream of the DraIII site. All primers contain additional 6 – 8 base pairs overhang for optimal restriction digest. The sequence for the 5' forward primer is: acacatagcgccggcgctagctgagcaaaaggccagcaaaaggcca. The sequence for the 3' reverse primer

15   is: aactctgtgagacaacagtcataaatgtacagatatcagaccaagtttactcatatatac. The PCR reactions are usually performed with proof-reading polymerases, such as Tth (PE Applied Biosystems), Pfu, PfuTurbo and Herculase (Stratagene), or Pwo (Roche), according to the manufacturer's recommendations. A typical PCR reaction for Herculase polymerase contains 1 μl template plasmid DNA (1-10 ng/ μl), 5 μl 10x buffer, 1 μl dNTPs (deoxynucleotide triphosphate) at

20   10 mM each, 1 μl forward primer (20 μM), 1 μl reverse primer (20 μM), 40 μl deionized, sterile water and 0.5 μl Herculase polymerase in a 50 μl reaction. The PCR reaction is performed at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds per cycle, for a total of 25 cycles. The PCR products were purified with phenol/chloroform using Phase lock Gel™Tube (Eppendorf) followed by standard ethanol precipitation. The purified

25   PCR products were digested with the restriction enzymes NgoMIV and DraIII according to the manufacturer's recommendations (New England Biolabs, Inc.) and gel purified using the QiaExII gel extraction kit (Qiagen) according to the manufacturer's instructions.

          The Kanamycin resistant gene (transposon Tn903) was isolated by PCR from plasmid pACYC177 (New England Biolabs, Inc.) using standard known procedures. The

30   Kana^r gene is used for in vivo or in vitro studies. Alternative antibiotic resistant genes, such as ampicillin, tetracycline, and blasticidin resistant genes, can be used for in vivo or in vitro studies in a variety of cell cultures.

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The 5' PCR primers contain the DraIII cloning site and an additional single restriction site, AscI, downstream of it. The 3' PCR primers contain the NgoMIV cloning site. The 5'forward primer sequence is: ggcttctcacagagtggcgcgccgtgtctcaaaatctct. The sequence for the 3' reverse primer is: ttgctcagctagcgccggcgccgtcccgtcaagtcagcgt. The PCR

5   reactions, product purification and digest with DraIII and NgoMIV were performed as described above. About 20 ng of each of the two PCR products were ligated in a 20 µl reaction, containing 2 µl 10x buffer and 1U ligase (Roche). Amplification in E. coli was performed using standard procedures as described in Sambrook, *supra*. Plasmids were purified with the QiaPrep-spin Miniprep kit (Qiagen) following the manufacturer's

10   instructions and digested with BsrG1 and DraIII for subsequent ligation of the mammalian transcription unit (promoter and polyA).

2.   Expression Vector pMaxVax10.1.

In this example, the CMV Towne promoter was used for driving the expression of the transgene in mammalian cells. Alternatively, other CMV promoters or

15   non-naturally occurring recombinant or chimeric CMV promoters can be used; for example, a chimeric or recombinant promoter, including an optimized CMV promoter, as described in copending, commonly assigned PCT Application Serial No. US01/20123, entitled "Novel Chimeric Promoters," filed June 21, 2001 as LJAQ Attorney Docket No. 02-031910PC, can be used, which is incorporated herein by reference in its entirety for all purposes. Different

20   strains of CMV can be obtained from ATCC. Strains AD169 (VR-538; Rowe, W. (1956) Proc. Soc. Exp. Biol. Med. 145:794-801) and Towne (VR-977; Plotkin, S.A. (1975) Infect. Immun. 12:521-27) were isolated from human patients with CMV infections, while strains 68-1 (Asher, D.M. (1969) Bacteriol. Proc. 269:91) and CSG (Black, H. (1963) Proc. Soc. Exp. Biol. Med. 112:601) were isolated from Rhesus and Vervet monkeys, respectively.

25   Other viral promoters, e.g., from RSV and SV40 virus, and cellular promoters, such as the actin and SRα promoter, and the like, and other promoters known to those of skill in the art, confer ubiquitous transcription in mammalian cells as well. For cell type-specific transcription, the use of cell type-specific promoters, such as muscle specific, liver specific, keratinocyte specific, and the like, and others known to those of skill in the art can be used.

30   The CMV Towne promoter was isolated from DNA of the CMV virus Towne strain by commonly known PCR methods. The cloning sites EcoRI and BamHI were incorporated into the PCR forward and reverse primers. The EcoRI and BamHI digested PCR fragment was cloned into pUC19 for amplification. For construction of the

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vector pMaxVax10.1, the CMV promoter was isolated from the pUC19 plasmid by restriction digest with BamHI and BsrG1. The BsrG1 site is located 168 bp downstream of the 5' end of the CMV promoter start, resulting in a 1596 bp fragment, which was isolated by gel purified for subsequent ligation.

5   The polyadenylation signal from the bovine growth hormone (BGH) gene was used in this example. Other poly A signals, which work well in mammalian cells, include, e.g., poly A signal sequences from, e.g., SV40, Herpes simplex Tk, and rabbit beta globin, and the like, and others known to those of skill in the art. The BGH poly A was isolated from the pCDNA3.1 vector (Invitrogen) using commonly known PCR methods.

10   The 5' PCR forward primer contained additional 14 bp sequence comprising recognition sites for the restriction enzymes PmeI and BglII, which form part of the poly linker. The 3' reverse primer contains the restriction site DraIII for cloning to the minimal plasmid Col/Kana. The 5'forward primer sequence is: agatctgtttaaaccgctgatcagcctcgactgtgccttc. The 3'reverse primer sequence is: acctctaaccactctgtgagaagccatagagcccaccgca. The resulting

15   PCR product was diluted 1:100, and 1 μl was used as a template for a second PCR reaction with the same 3'reverse primer and a new 5' forward primer. This primer was overlapping the 5' end of the template by 20 bp and contained another 40 bp 5', containing BamHI, KpnI, XbaI, EcoRI and NotI recognition sequences to form the rest of the polylinker. The sequence of the 5'extension primer is:

20   ggatccggtacctctagagaattcggcggccgcagatctgtttaaaccgctga. An alternative PCR product was generated with different 5'forward PCR primers to generate a vector with a modified polylinker, designated pMaxVax10.1mp (Fig. 21 with modified polylinker as described above). The orientation of the restriction sites in this polylinker is 5'-3': BamHI, XbaI, KpnI, EcoRI, NotI, BglII, and PmeI. The polylinker sequence is:

25   ggatccactcatctagaacaatggtaccaatacgaattcggcggccgcagatctgtttaaacc. The PCR products were digested with BamHI and DraIII and gel purified.

           The final ligation reaction contained about 20 ng each of the BsrG1 and BamHI digested CMV promoter, of the BamHI and DraIII digested polylinker and BGH poly A, and the DraIII and BsrG1 digested minimal plasmid Col/Kana in a 50 μl reaction

30   with 5 μl 10 x ligase buffer and 2U ligase (Roche). Ligation, amplification and plasmid purification were performed as described above.

      B.   <u>Construction of Vector pMaxVax with NCSM Polynucleotide Sequence</u>

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The nucleotide sequence encoding a NCSM polypeptide (e.g., a CD28BP or CTLA-4BP polypeptide or fragment thereof, such as an ECD domain) or any other immunomodulatory molecule can be isolated by PCR with BamHI and KpnI restriction enzyme recognition sequences in the PCR forward and reverse primer as described above.

5   In this example, a polynucleotide sequence encoding a CD28BP polypeptide (e.g., CD28BP-15 polypeptide (SEQ ID NO:19) is incorporated into the pMAxVax 10.1 vector. To verify the correct sequence of the PCR products, the fragments are cloned conveniently into the TOPO® cloning vectors (Invitrogen) for sequencing according to the manufacturer's protocols. After BamHI and KpnI digestion and gel purification, the genes

10   are cloned into a mammalian expression vector to confirm the expression of the gene. To clone the genes into the polylinker of pMaxVax, the vector pMaxVax 10.1mp (Fig. 21 with modified polylinker as described above) was digested with BamHI and KpnI, gel purified and ligated to the respective genes, as described above. The construct pMaxVax-CD28BP (see Figure 22A), which includes the nucleotide sequence encoding a CD28BP (here, e.g.,

15   SEQ ID NO:19), can be used for in vivo and in vitro expression in human and other mammalian cells and other cells in culture, including non-mammalian cells and the like.

For in vitro expression the immune stimulatory molecules can also be cloned in any commercially available vectors such, as pCDNA3.1+/-, pCDNA4 (Invitrogen), which are suitable for stable expression under drug selection in mammalian cells. If secretion is a

20   desired feature, the genes can be cloned into vectors such as pSecTag, pDisplay, pBC1 (Invitrogen), which link the expressed proteins to secretion signals. For regulated expression vectors from the Tet™System (Clontech) or Ecdysone regulatory vectors (Invitrogen) can be used. For high expression levels in cell culture the immune stimulatory molecules can also be cloned into viral vectors constructed from Retrovirus, Adenovirus

25   (Clontec), and Sindbis virus (Invitrogen), or replicating viral vectors constructed from EBV, BPV, HPV and SV40 virus. For in vivo studies, viral vectors constructed from Adenovirus, Lentivirus, and Alphaviruses, and the like can be used. If restriction sites other than BamHI and KpnI are required for cloning into the different vectors, flanking restriction sites from the polylinker can be used. Alternatively, the genes can be isolated by PCR with the desired

30   restriction sites located in the PCR primers as described above.

C.   Bicistronic vector pMaxVax-CD28BP-EpCAM/KSA.

For immunotherapy studies it is desirable to express the immunostimulatory molecule in the same cells as, for example, a cancer antigen. A nucleotide sequence

<p style="text-align:center">243</p>

encoding a cancer antigen, such as EpCam/KSA or a mutant or variant thereof, can be cloned into the pMaxVax vector (Fig. 21) to generate a pMaxVax-EpCam/KSA vector, using a procedure analogous to that described above for cloning the CD28BP polynucleotide sequence into the pMaxVax vector backbone. Two expression constructs,

5   e.g., the pMaxVax-CD28BP vector (Fig. 22A) (or other pMaxVax-NCSM vector) and the pMaxVax-EpCam/KSA vector (or other pMaxVax vector including a nucleotide sequence encoding an antigen), can then be co-transfected in cell culture or co-administered in vivo to a subject in need of such therapeutic or prophylactic treatment.

In an alternative format, which may be an optimal format for some

10   therapeutic or prophylactic applications, both the EpCam/KSA (or a EpCam/KSA mutant or variant thereof) and CD28BP genes (or a different antigen gene and/or NCSM polynucleotide) can be expressed from the same vector. In one format, the resulting antigen and NCSM proteins can be co-expressed from a single promoter linked by an internal ribosomal entry site (e.g., IRES bicistronic expression vectors, Clontec). This example

15   describes the construction of an exemplary bicistronic vector for expression of at least one NCSM polypeptide and at least one antigen or antigen fragment (or a different co-stimulatory molecule) in which the NCSM polynucleotide and the nucleotide sequence encoding the antigen or antigen fragment form two separate expression units. In particular, this example describes the construction of a bicistronic vector for expression of CD28BP

20   (e.g., CD28BP-15) and the cancer antigen EpCAM/KSA (or mutant or variant thereof) in which the CD28BP polynucleotide and the polynucleotide encoding the cancer antigen or antigen fragment form two separate expression units, each regulated by its own respective promoter and poly A signal. One of skill will understand that this procedure can also be readily adapted to construct a bicistronic vector comprising at least one NCSM

25   polynucleotide of the invention (including nucleic acid fragments thereof, and nucleic acids encoding soluble NCSM polypeptides, peptide fragments thereof, and fusion proteins thereof described herein) and a different antigen or antigen fragment (or a different co-stimulatory molecule).

The CD28BP gene is inserted into the polylinker of a pMaxVax vector as

30   described above, forming the first expression unit. The nucleic acid sequence of the cancer antigen, here the polynucleotide encoding the extracellular domain of EpCAM/KSA (or mutant or variant thereof), is linked to a second mammalian expression promoter (exemplary promoters include those set forth in this Example above and elsewhere) and a

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second poly A signal (exemplary signals include those set forth in this Example above and elsewhere) to form the second expression unit. In this Example, a synthetic poly A (SPA) sequence was made and used. However, one of skill in the art would understand that other poly A sequences (e.g., bovine growth hormone (BGH) poly A or SV40 poly A sequence)

5 can also be used. The synthetic poly A was derived from a sequence for the rabbit β-globin poly A (Gen&Dev. 3:1019-1025 (1989). The sequence fragment was generated by annealing two oligonucleotides, which contained the respective cloning sites in the 5' and 3' sequences. The upper strand sequence is: 5'-GATCTGTTTAAACTCTGGCTAATAAAAGATCAGAGCTCTAGACATCTGTGTGTT

10 GGTTTTTTGTGTGTCTCACTCACAGA-3', and the sequence of the lower oligonucleotide strand is: 5'-TGAGTGAGACACACAAAAAACCAACACACAGATGTCTAGAGCTCTGATCT TTTATTAGCCAGAGTTTAAACA-3".

The second expression unit can be cloned into 3 different sites in the

15 construct pMaxVax-CD28BP, both in forward or reverse orientation: (i) downstream of the first expression unit (e.g., CMV promoter-CD28BP-SPA polyA, CMVpromoter-CD28BP-BGH polyA, or CMVpromoter-CD28BP-SV40 polyA) using the single cloning sites DraIII and AscI in pMaxVax10.1; (ii) between the ColE1 and Kana^r gene using the single restriction sites NgoMI and NheI; (iii) between the Kana^r gene and the CMV promoter into

20 the single EcoRV and BsrGI restriction sites (see vector description above in this Example). Independent of the location of the second expression unit it is advisable to add a terminator sequence downstream of the first expression unit. A consensus terminator sequence 5'-ATCAAAA/TTAGGAAGA3' is described in Ming-Chei Maa et al. (1990) JBC 256 (21):12513-12519. In the construct pMaxVax,CD28BP the sequence can be placed into the

25 single DraIII site downstream of the poly A sequence (e.g., synthetic poly A or SPABGH poly A sequence) (see Figure 22B).

This example describes the cloning strategy of the second expression unit for location (ii). The second promoter (e.g., a WT CMV promoter, such as human CMV promoter or a recombinant CMV promoter with improved expression activity), the

30 EpCAM/KSA cancer antigen (or mutant or variant thereof), and the second poly A (in the example, synthetic poly A or SV40 polyA), are isolated from the respective template plasmids by PCR or assembled from oligonucleotides (as described above in this Example). The PCR primers are designed to contain single restriction sites, which allow for partial

site-directed cloning of the three fragments into the final vector. The 5'forward PCR primer

for isolation of the shuffled CMV promoter contains the single NgoMIV (also called

NgoMI) cloning site. The 3'reverse primer contains the NgoMIV site and another restriction

enzyme site, which does not cut in any of the other vector units (i.e. AccI, AgeI, AvrII,

5   BsU361, MluI, RsrII, SalI) upstream of it separated by a spacer of at least 10 base pairs. In

the example AccI is chosen as the additional cloning site. The PCR product is digested with

NgoMIV followed by gel purification and cloned into the NgoMIV linearized and gel

purified pMaxVax,CD28BP. The correct orientation of the second CMV promoter after

ligation is determined by PCR from bacterial colonies (as described in Molecular Cloning,

10 A Laboratory Manual, Sambrook and Russell) using the 3'reverse primer and any forward

primer of choice located about 500 – 600 bp upstream of the reverse primer in the CMV

promoter sequence. The second promoter containing plasmid is then digested with AccI

and NheI for cloning of the cancer antigen. The 5'primer for the EpCAM/KSA cancer

antigen (or mutant or variant thereof) contains the single AccI site and the 3'primer the

15 single NheI site and an additional single restriction site upstream, AgeI, separated by a

spacer of at least 10 base pairs. The PCR product is digested with the enzymes AccI and

NheI and cloned into the equally digested vector. The resulting construct is digested AgeI

and NheI for cloning of the SV40 polyA/terminator fragment. The 5' forward primer for

this PCR product contains the single AgeI site and the 3'reverse primer the terminator

20 sequence followed by the single NheI site. The 5' cloning sequence and the NheI site are

incorporated in the oligonucleotides. The resulting (e.g., double-stranded) AgeI / NheI poly

A fragment is then cloned in the equally digested vector. The cloning strategy is outlined

below.

1) NgoMIV-----------------------AccI/NgoMIV
25   CMV promoter
                                    AccI
    2)   AccI--------------------AgeI/NheI

            Ep-CAM/KSA

30 3)   AgeI-------------NheI

                                        synthetic poly A or SV40polyA)

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An exemplary construct pMaxVax,CD28BP,EpCAM/KSA is shown in Figure 22B.

One of skill will understand that a similar procedure can be used to construct an expression vector comprising a nucleotide sequence encoding a CTLA-4BP of the invention (in place of the sequence encoding CD28BP above in Fig. 22A. Such a vector can comprise a bicistronic vector, if desired, with a second nucleotide sequence of interest (e.g., encoding an antigen or another co-stimulatory molecule) included in the position occupied above by the antigen, as shown in Fig. 22B. One of skill will also understand the above procedure can be readily adapted to construct an expression vector comprising different vector components, such as different promoters, signal sequences, termination sequences, replication origin sequences, resistant gene or marker sequences.

EXAMPLE VI: Enhanced Immune Response Induced by a CD28BP Polypeptide

This example demonstrates the ability of a CD28BP molecule of the present invention (or fragment thereof) to enhance an immune response of a heterologous antigen, such as a tumor-associated antigen (Ag), such as, e.g., Ep-Cam/KSA (as described in Strand et al. (1989) Cancer Res. 49:314-317; Szala et al. (1990) 87:3542-3546; Balzar et al. (1999) J Mol Med 77:699-712), or a polypeptide variant, mutant, or derivative of EpCam/KSA polypeptide (or a nucleotide sequence variant, mutant, or derivative encoding such EpCam/KSA polypeptide variant, mutant or derivative, respectively), or a pathogen antigen (e.g., hepatitis B surface Ag (HepBsAg)), in cynomolgus monkeys. The EpCam/KSA polypeptide variant or mutant may comprise, e.g., an amino acid sequence of EpCam/KSA in which at least one amino acid has been replaced by another amino acid; the substitution may comprise a conservative amino acid substitution. The corresponding nucleotide sequence may comprise, e.g., a substitution of one or more nucleotide residues such that the EpCam/KSA polypeptide variant or mutant amino acid sequence is encoded therefrom. In this example, a vector comprising a nucleotide sequence encoding full-length clone CD28BP-15 is used. If desired, alternatively a vector comprising a nucleotide sequence encoding a fragment of CD28BP-15 (e.g., such as an ECD) or encoding a fusion protein (e.g., ECD-Ig) can be used. For example, a sequence encoding a soluble NCSM of the invention (e.g., CD28BP-15ECD, CD28BP-15-ECD-Ig, or with a trunECD, or the like) can be used. A vector comprising a nucleotide sequence encoding a WT hepatitis B surface antigen (hepBsAg) (or fragment thereof) and a vector comprising a nucleotide sequence

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encoding Ep-Cam or EpCam variant or mutant (or a fragment of any of these) is used as the antigen sequence. The procedure can be adapted to use any NCSM molecule described herein and/or any antigen of interest, including, e.g. viral antigens or other cancer antigens described infra.

5   In the following example, separate vectors are prepared that encode each of EpCam (or mutant or variant thereof), WT hB7-1, CD28BP-15, and the antigen. A separate control vector is also prepared. See Example V. However, as noted below and as described in Example V, a bicistronic vector encoding antigen (e.g., EpCam or a mutant or variant polypeptide sequence thereof) and B7-1, or encoding antigen (e.g., EpCam or mutant or

10   variant polypeptide thereof) and CD28BP-15 can be used alternatively. If desired, a vector comprising a nucleic acid sequence encoding a fragment of an NCSM polypeptide having the desired properties can be used, such as a nucleic acid sequence encoding a signal sequence (of SEQ ID NO:66 or another NCSM molecule or from B7-1) and the ECD of CD28BP-15 (of SEQ ID NO:66), wherein the ECD has a CD28/CTLA-4 binding affinity

15   ratio that is at least about equal to or greater than that of B7-1 and/or induces a T cell response that is greater than that induced by B7-1. Vectors comprising sequences encoding other antigens and/or NCSM molecules, cytokines, costimulatory sequences, and the like or other vector elements can be constructed by using the vector construction procedures described above. One of skill will readily understand how to modify/adapt these procedures

20   to construct vectors comprising nucleotide sequences encoding such NCSM molecules with or without also encoding any of such antigens.

In this analysis, five groups of cynomolgus monkeys (3 monkeys per group) are inoculated intradermally (i.d.) (e.g., by a gene gun or injection with a needle) or intramuscularly using DNA plasmid expression vectors with either CD28BP-15 alone,

25   hB7.1 alone, antigen (Ag) alone (such as, e.g., EpCam/KSA or a mutant or variant thereof), CD28BP-15 with Ag or hB7.1 with Ag, each at a total dose of 1 milligram DNA per inoculation as outlined in Table 8 below. For CD28BP-15, the vector typically comprises a nucleotide sequence encoding SEQ ID NO:19 or a nucleotide sequence encoding the polypeptide of SEQ ID NO:66. A DNA plasmid control vector lacking a nucleic acid insert

30   encoding a CD28BP-15, hB7-1, or Ag is used to equalize the total amount of DNA used in each injection. Procedures for constructing the pMaxVax plasmid vector alone (control vector) and a plasmid vector comprising a nucleotide sequence encoding CD28BP-15 are described in Example V above. Similar procedures can be used to construct a separate

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pMaxVax vector or the like comprising a nucleotide sequence encoding a human B7-1, Ag, or HepBsAg, as shown in Table 8. Alternatively, another plasmid-based mammalian expression vector or a viral vector can be employed in the following procedure, including any of those described above in the specification. Immunized animals are monitored daily for any local and systemic reactions.

Table 8

Group	No. of animals	Immunization	Dose (mg DNA) for each vector
1	3	CD28BP-15 vector+Control vector	0.5+0.5
2	3	HB7-1 vector + Control vector	0.5+0.5
3	3	Ag vector + Control vector	0.5+0.5
4	3	CD28BP-15 vector + Ag vector	0.5+0.5
5	3	HB7-1 vector + Ag vector	0.5+0.5

Animals. Five groups of 3 male Cynomolgus monkeys, each weighing approximately 4 kg (15 total), are used. Animals are randomly assigned to groups using a number draw. In addition, each animal is assigned a specific number within that group.

Inocula. Mixtures of plasmid DNA to contain 0.5 mg of each (separate) vector component as outlined in Table 8 are prepared. Total plasmid DNA delivered is 1 mg in each case. Each DNA expression plasmid is diluted in PBS, pH 7.4 from a stock solution to achieve the target concentration in 1 ml per inoculum.

(Alternatively, a bicistronic format is used in which the following plasmid vectors are made and substituted in the procedure: 1) a plasmid vector comprising a nucleotide sequence encoding EpCam or mutant or variant polypeptide thereof (total DNA plasmid dose is 1 mg) (antigen control vector); 2) a plasmid vector comprising a nucleotide sequence encoding both EpCam (or mutant or variant thereof) and WT hB7-1 (total DNA plasmid dose is 1 mg) (antigen/WT hB7-1 control vector) (biscistronic vector that co-expresses Ag and hB7-1); 3) a plasmid vector comprising a nucleotide sequence encoding both EpCam (or mutant or variant thereof) and CD28BP-15 (or fragment thereof, including soluble form) (total DNA plasmid dose is 1 mg)(bicistronic vector co-expressing EpCam(or mutant or variant thereof), and CD28BP-15). The bicistronic vectors are prepared as described for the pMaxVax bicistronic vector encoding both a CD28BP and EpCam (or mutant or variant thereof) in Example V above.

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Inoculation. Animals are anaesthetized prior to inoculation. The backs of the animals are first prepared by shaving the fur and the animals are inoculated by i.d. or i.m. injection with 1.0 ml of a total 1 mg DNA plasmid vector(s) as described in Table 8 above (or alternative amounts described below) at multiple sites. The monkeys are boosted three times at 3 weekly intervals with the same inoculation dose or with 0.1 mg Antigen protein (e.g., EpCam polypeptide or a polypeptide mutant or variant thereof).

Observation and monitoring. Each inoculation site is examined every day, beginning at day 1, for any delayed-type hypersensitivity (DTH) reaction. Animals are observed daily for signs of systemic reaction to the inoculation. These observations include, but are not limited to, changes in weight, body temperature, eating habits, skin and hair, eyes, mucous membranes, respiratory system, circulatory system, central nervous system, somatomotor activity, elimination, behavior, and any occurrence of tremors, convulsions, salivation, diarrhea, lethargy, or coma.

Collection of blood. Monkeys are bled to obtain 2-5 ml of whole blood one day prior to immunization and weekly thereafter. Blood is allowed to clot, serum separated, frozen at -20°C until further analysis. On alternate weeks, however, 5-10 ml of blood is drawn in heparinized tubes for T cell assay analysis.

Tissue collection. Punch biopsies of the inoculation site are taken according to standard known procedures once every three weeks.

Sample analysis. Antibody titers against each of Ep-Cam (or mutant or variant thereof) and HepBsAg in the sera of the animals are determined, respectively, using ELISA assays (Mosolits et al. (1999) Cancer Immunol. Immunoth 47:315-320; Staib et al. (2001) Intl. J. Cancer 92:79-87; Chow et al. (1997) J. Virol. 71:169-178). Furthermore, T cell proliferation in response to one of these antigens is analyzed by adding 10 g/ml of the antigen to cultures of 10^5 peripheral blood monocyte cells (PBMC). The cells are incubated for 3 days and incorporation of ^{3}H-thymidine during the last 8 hours of culture is measured by scintillation counting (as described in Punnonen et al. (1994) J. Immunol. 152:1094-1102). See T cell proliferation methods described above. A higher T cell proliferative response indicates a more vigorous immune response as a result of the vaccination.

Cytokine production, such as, e.g., IFN-gamma, IL-2, IL-4, IL-5, IL-12, and IL-13 production, is studied in response to the specific antigen using cytokine specific ELISAs (R&D Systems) or ELISpot assays (Biosource International, Camarrillo, CA), performed according to the manufacturer's instructions. For example, enumeration of IFN-

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gamma secreting cells in single cell suspension is performed using a kit obtained from Biosource International (Camarrillo, CA) (see manufacturer's instructions). The following protocol is used. 50 μl of diluted coating antibody is added to each well followed by the addition of 50 μl of PBS. Each well is incubated overnight at 4°C. Samples are then

5   aspirated and washed 5 to 10 times with wash buffer. 200 μl of post-coating solution is added into each well and wells are incubated 1 hour at 37°C or overnight at 4°C. The wells are aspirated and not washed. Wells are 100 μl of prestimulated single cell preparation are added into the wells. The plate is covered with the plate cover and incubated for 5 hours at 37°C in a humidified atmosphere containing 7% CO_2. The wells are aspirated, 200 μl ice-

10   cold deionized water is added, and the plate is placed for 10 min on melting ice. The wells are washed 10 times with PBS. 100 μl of diluted biotinylated Antibody solution is added, the plate is covered and incubated for 1 hour at 37°C or overnight at 4°C. The wells are aspirated and washed 5 to 10 times with PBS. 50 μl of diluted - labeled anti-biotin antibody solution (GABA) is added to each well. The plate is covered and incubated 1 hour at 37°C.

15   The wells are aspirated and washed 5 to 10 times. 30 μl of activator solution is added to each well. The spot development is followed by light microscopy. When clear spots have developed, the reactions are stopped by rinsing the wells with distilled water. The results are compared between animals immunized with the antigen with or without CD28BP-15.

Such plasmid expression vectors encoding CD28BP-15 with and without an

20   antigen are useful in therapeutic and prophylactic treatment protocols as described above. Plasmid expression vectors encoding CD28BP-15 and EpCam/KSA (or mutants or variants of EpCam polypeptide or nucleotide) are useful in methods for therapeutically and/or prophylactically treating a variety of cancers, as described above. Given that the primate model is an accepted model closely related to human, such methods may be readily adapted

25   by one of ordinary skill in the art to therapeutic and/or prophylactic vaccination protocols for humans.

A similar procedure to that described above can be employed to assess an ability of a CTLA-4BP of the invention to inhibit an immune response or inhibit T cell proliferation or CTL responses in a subject, by substituting a nucleotide sequence encoding

30   a CTLA-4BP of the invention in place of the nucleotide sequence encoding CD28BP-15 and using the functional assays for, e.g., T cell activation. For example, T cell activation can be analyzed by measuring proliferation, cytokine production, CTL activity or expression of activation antigens such as IL-2 receptor, CD69 or HLA-DR molecules, as described above.

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Vectors that harbor CTLA4-BP genes that efficiently act through CTLA-4 are useful in inducing, for example, tolerance and anergy of allergen- or autoantigen-specific T cells. In some situations, such as in tumor cells or cells inducing autoimmune reactions, the antigen may already be present on the surface of the target cell, and the vectors encoding CTLA-

5   4BP molecules may be transfected in the absence of additional exogenous antigen gene.

Boosting. In methods described herein using either separate vectors encoding each of Ag, hB7-1 or CD28BP, or bicistronic vectors encoding Ag and CD28BP, or Ag and hB7-1, one or more additional doses of DNA plasmid vector (e.g., 1 mg) can be administered subsequently to an animal at one or more subsequent intervals (e.g., 2 times),

10   respectively enhance or "boost" the immune response. If desired, following a boosting of the immune response with such administration of one or more additional DNA plasmid vector doses, at least one dose of the EpCam protein (protein dose of from about 0.1 to about 1 mg) ("protein boost) (or EpCam mutant or variant) can be administered to an animal to further enhance or "boost" the immune response. Additional protein boosts can be

15   administered subsequently at desired intervals (e.g., after one or more days, one or more weeks, one or more months).

EXAMPLE VII: Blocking Development of EAE

The mouse model of Experimental Autoimmune Encephalomyelitis (EAE)

20   has many similarities with human multiple sclerosis (MS), and it has been widely used as a model of human MS (see, e.g., Alvord, G.C. Jr., ed., Experimental Allergic Encephalomyelitis: A Useful Model for Multiple Sclerosis, Liss, N.Y. (1984)). EAE can be induced in SJL/F mice by myelin basic protein (MBP) or proteolipid-protein (PLP) or peptides thereof.

25   To demonstrate the efficacy of a CTLA-4BP molecule of the invention to prevent EAE, the following prophylactic treatment vaccination protocol is used. DNA expression plasmids encoding either CTLA-4BP or MBP (or PLP) are codelivered, or the two genes for CTLA-4BP and MBP (or PLP) are coexpressed in the same vector and delivered, as follows. In this example, a nucleotide sequence encoding clone CTLA-4BP

30   5x4 12c is used. Procedures for constructing the pMaxVax plasmid vector alone (control vector) or with a nucleotide sequence encoding a CD28BP and/or a second polypeptide (EpCam or mutant or variant thereof) are described in Example V above. One of skill can readily adapt such procedures to construct pMaxVax vectors comprising the nucleotide

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sequence encoding a CTLA-4BP and/or MLP (or PLP), expressed alone on separate vectors or coexpressed on one vector. Alternatively, another plasmid-based mammalian expression vector or a viral vector can be used in the following procedure, including any of those described above in the specification. 100 μg of the DNA plasmid in 100 μl PBS is injected intramuscularly or intradermally to SJL/F female mice. A control DNA plasmid lacking the CTLA-4BP, MBP, or PLP nucleotide sequence is similarly administered to a control group of mice.

To induce EAE, mice are injected intradermally with 100 μl rabbit brain myelin basic protein (MPB) at 1 mg/ml in complete Freund's adjuvant. Mice are analyzed for the onset of EAE by visually noting tail paralysis followed by hind leg paralysis (at which point animals are sacrificed for humane reasons).

The ability of a DNA plasmids encoding CTLA-4BP and/or MBP (or PLP) to block EAE is demonstrated by the number of mice developing EAE and the severity of the disease, as compared to mice that received the control DNA plasmid.

EXAMPLE VIII: Improved Cell-Based Vaccines for the Treatment of Cancer

To enhance the immunogenicity of tumor cells used as cell-based vaccines for the immunotherapeutic or prophylactic treatment of a variety of cancers, patient tumor cells can be transfected with a CD28BP nucleic acid (NA) sequence of the present invention. In this example, the sequence corresponding to clone CD28BP-15 is used; however, other NA sequences of the invention can be readily employed. As an example, the specific immunotherapy involves immunization of melanoma patients with a polyvalent, irradiated whole cell melanoma cells transfected with a DNA plasmid encoding CD28BP-15.

In one such method, a population of tumor cells derived from a melanoma patient's melanoma tumor cell lines (i.e., cells removed from the patient) are transfected (e.g., by electroporation) with a sufficiently effective amount of DNA expression plasmid vector, pMaxVax, encoding CD28BP-15 (or fragment thereof, e.g., CD28BP-15-ECD or expressed soluble CD28BP) that facilitates uptake and expression of CD28BP-15 polypeptide on the cells; the amount of DNA plasmid typically constitutes a therapeutically or prophylactically effective amount or dosage to treat the melanoma cancer or prevent further development of the cancer. The pMaxVax plasmid is described in example V

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above. Or, another plasmid-based mammalian expression vector, or viral vector, can be used in this procedure, including those described herein and throughout.

These transfected tumor cells are inactivated by irradiation (50 gray) and cryopreserved for used as the cell-based vaccine. Prior to treatment (delivery to the patient), the cells to be used as vaccine are thawed and washed 3 times in phosphate-buffered saline; if desired, the cells to be used as a vaccine are formulated as a composition with an excipient, such as, e.g., a pharmaceutically acceptable excipient, e.g., PBS. (In an alternative format, allogeneic melanoma tumor cells are transfected with a sufficient amount of pMaxVax DNA plasmid vector encoding CD28BP-15 (or a fragment thereof, e.g., CD28BP-ECD) for CD28BP-15 expression.) Transfected tumor cells encoding an effective amount of expressed CD28BP-15 (or a composition comprising such cells)--either those derived from the specific patient's cell line or allogeneic cells--are injected intradermally into the specific patient in auxiliary and inguinal regions in escalating doses once every 2 weeks for 3 months. The first and second injections of the vaccine comprise 2×10^6 cells, followed by 6×10^6 cells for the third and fourth injections, and then 18×10^6 cells for the fifth and sixth injections.

Immune responses of each patient are analyzed by measuring the levels of tumor cell specific Abs and the level of T cell response against the antigen or antigenic fragment expressed on the tumor cells by analyzing T cell proliferation in response to tumor cell lysates and measuring delayed type hypersensitivity (DTH) reaction. T cell response against the cancer antigen is analyzed using standard methods described above (see, e.g., Example VI). Levels of tumor cell specific Abs in the patients' sera are measured by ELISA using standard protocols (see Colligan; Sambrook; Rapley and Walker, all *supra*). To analyze DTH, tumor cell lysates are injected intradermally into the back of patients. Responses are evaluated on days 1, 2, 4, and 7 after injection. The mean diameter of induration is calculated as (greatest diameter + perpendicular diameter)/2. A positive response is defined as a mean diameter of induration of 5 mm. Four-millimeter punch biopsies of positive reactions are performed on selected consenting subjects to analyze the phenotype of infiltrating cells using flow cytometry (FACSCalibur flow cytometer and CellQuest software, BDIS) as described above. The single cell suspensions are then stained anti-CD3, CD4, CD8, CD14, and CD20 monoclonal antibodies to measure the percentages of T cells, CD4+ T helper cells, CD8+ cytotoxic T cells, monocytes and B cells, respectively.

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Estimated statistical survival rates are analyzed by the non-parametric Kaplan-Meier method (see Kaplan et al., J Am Stat Assoc (1958) 53:457) (e.g., using the statistical analysis software JMP (ver. 3.1 for Macintosh; SAS Institute Inc., Cary, NC)). The log-rank test is used to determine the differences in survival of patients from subgroups

5   defined by different levels of risk factors. Survival times are defined as the length of time a given patient remains alive after the diagnosis of metastatic disease to either a regional site (AJCC Stage IIIA), with regard to skin and soft tissue metastasis, or a distant site (AJCC Stage IV).

10   EXAMPLE IX: B7-1 Polypeptide Variants with Altered Properties

Sequence analysis of CTLA-4BP molecules exhibiting preferential binding to CTLA-4 relative to CD28 revealed that many such molecules showed a substitution of another amino acid for tyrosine at the amino acid position corresponding to amino acid position 65 of the full-length amino acid sequence of hB7-1 (SEQ ID NO:278). These data

15   suggested the amino acid residue at this position played a different role in the binding of these molecules to CTLA-4 than to CD28. A polypeptide variant of the hB7-1 polypeptide shown in SEQ ID NO:278 was made which comprised a substitution of histidine for tyrosine at position 65 of the amino acid sequence of SEQ ID NO:278, where the amino acid position is measured from the N-terminus. Position 65 corresponds to position 31 of

20   the mature domain of hB7-1 (SEQ ID NO:278), as measured from the N-terminus. Specifically, codon TAC of the nucleic acid sequence of hB7-1 shown in SEQ ID NO:273, corresponding to the three nucleic acid residues at positions 193-195 of SEQ ID NO:273, was mutated to codon CAC. Digestion of the expression plasmid encoding hB7-1with Pml I and Xcm I removed a 200 base pair (bp) fragment which included the amino acid at

25   position 65 encoding Tyr (Tyr65). Using standard molecular biology cloning methods, this nucleotide fragment was replaced with a corresponding Pml I – Xcm I DNA nucleotide fragment (200 bp) from the nucleic acid sequence encoding CTLA-4BP 5x4-12c, which encoded histidine at the amino acid position corresponding to position 65 of hB7-1 (SEQ ID NO:278). The remaining amino acids encoded by the 200-bp fragment were otherwise

30   identical between hB7-1 and CTLA4BP 5x4 12c. The B7-1 polypeptide variant (termed "hB7-1-Tyr65His polypeptide variant") comprised the full-length sequence of SEQ ID NO:278 with a Tyr65His substitution (mutation). Methods for detecting and/or measuring

expression, binding to CD28-Ig and/or CTLA-4-Ig, and T-cell proliferation were performed as described above in previous Examples.

Figures 23A-23B show that the hB7-1-Tyr-65His polypeptide variant binds to soluble CTLA-4-Ig more preferentially than it does to soluble CD28-Ig. Figures 23A-23B are histograms depicting the differential binding of full-length hB7-1-Tyr65His variant, CTLA-4BP 5x4-12c (gray histogram), and hB71 gray histogram) to either soluble CD28-Ig or CTLA-4-Ig. The binding levels are analyzed by flow cytometry (FACS) as described previously. HEK 293 cells (2×10^5 cells) transfected with either the nucleic acid sequence encoding hB7-1 (SEQ ID NO:278), CTLA-4BP 5x4 12c, or hB7-1-Tyr65His polypeptide variant were incubated with 2.5 microgram/milliliter (ug/ml) CTLA-4-Ig or 30 ug/ml CD28-Ig (both of which are saturating concentrations), and binding was determined by FACS analysis. Panel A shows that 293 cells expressing hB7-1, CTLA-4BP 5x4-12c, or hB7-1-Tyr65His polypeptide variant bind CTLA4-Ig in a substantially similar manner. Untransfected or pCDNA3.1 transfected 293 cells showed no binding to CDLA-4-Ig. However, as shown in Panel B, the binding of each of hB7-1-Tyr65His variant and CTLA-4BP 5x4-12c polypeptide to CD28-Ig was dramatically reduced to a level approximating that of untransfected or pCDNA3.1 transfected 293 cells. Cells expressing hB7-1 showed strong binding to CD28-Ig.

In addition to evaluating ligand binding, 293 HEK cells transfected with pCDNA or with a nucleic acid sequence encoding hB7-1 (SEQ ID NO:278), hB7-1-Tyr65His polypeptide variant, CTLA-4BP 5x4-12c (SEQ ID NO:39) (designated as clone 12c), or CD28BP-15 (SEQ ID NO:19), , and 293 cells alone (with no DNA) were assayed for their ability to induce human T-cell proliferation (see Figure 24). A null response was observed with the negative controls (293 cells transfected with pCDNA and 293 cells without any DNA) and a robust proliferative response was observed with CD28BP-15 – about 3-fold higher than cells expressing hB7-1. Cells expressing the hB7-1-Tyr65His variant and CTLA4-BP 5x4-12c showed proliferation responses virtually identical to those of the negative control groups, indicating that T cells received little or no positive co-stimulatory signal. The lack of T cell induced proliferation by the hB7-1-Tyr65His variant and CTLA-4BP 5x4-12c transfectants was not due to a lack of cell surface expression of these molecules, since binding of these molecules to CTLA4-Ig was normal (see inserted histogram in Fig. 24). The data suggest the Tyr65His substitution in hB7-1 is a substitution (mutation) that results in a polypeptide variant that preferentially binds to CTLA-4 relative

to CD28 and/or induces a decreased level of T cell proliferation or does not induce T cell proliferation compared to the level of T cell proliferation induced by hB7-1, as determined by T cell proliferation analyses, under the conditions set forth in Example IX (Figs. 23-24). The polypeptide variant did not bind CD28-Ig (Figs. 23A-23B) as did hB7-1, but it did bind CTLA-4-Ig in a manner that is substantially identical or equivalent to that of hB7-1; the binding profile of the polypeptide variant for CTLA-4-Ig was substantially identical or equivalent to that of hB7-1. The polypeptide variant has a CTLA-4/CD28 binding affinity ratio that is about equal to or greater than the CTLA-4/CD28 binding affinity ratio of a hB7-1, under the conditions described in Figs. 23-24.. Substitution of an amino acid that would constitute a conservative substitution of His for Tyr at this position of hB7-1 or substitution of an amino acid different than His, but having functional or chemical properties similar to His (Arg, Lys, Pro, Phe, and/or Trp), may produce variants with similar properties.

EXAMPLE X: Molecules with Immunosuppressive Properties

CTLA-4-Ig has been used as an antagonist of CD28 signaling, because the molecule binds to B7-1 and B7-2 with high affinity and thereby prevents the interaction of B7-1 and/or B7-2 with CD28 expressed on T cells. CD24-Ig also has been used in clinical trials to treat psoriasis and has been shown to provide clinical benefits. To compare CTLA-4-Ig with CD28BP-15-ECD-Ig and CD28BP-15-ECD with regard to immunoregulation, we examined the effects of these molecules on an antigen specific memory T cell response. The ECD of CD28BP-15 (clone 15) comprises the amino acid sequence of at least about amino acids 35-244 of SEQ ID NO:66.

Blood samples from three donors were obtained from Stanford blood bank (Stanford, CA) (Figures 25A-25C). PBMCs were isolated from each of these donors' blood by centrifugation over Histopaque-1077 (Sigma). PBMCs were used at 1×10^5 cells/well in 96-well round bottom assay plate and 1ug/ml of Tetanus Toxoid (TT, Calbiochem, San Diego, CA) was added into the cultures. Increasing concentrations (ug/ml) of soluble proteins of CD28BP-15-ECD-Ig (open triangle), CD28BP-15-ECD (close triangle), WT huB7.1-ECD-Ig (close square), WT huB7.1-ECD (open square), commercial CTLA-4Ig (R&D Systems) (closed diamond), and control human IgG antibody (open circle) were added to the cultures as described above. A non-specific human IgG was used as a negative control in this Tetanus Toxoid specific response. Assay plates were incubated for total of 5 days in 200ul of Yssel's medium. 1uCi/well of ^{3}H-thymidine was added during the last 8

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hours of incubation prior to being harvested. ^{3}H-thymidine incorporation is indicative of T cell proliferation as described previously. The data represent a mean of C.P.M.; each data point was calculated from triplicate wells. The immunosuppressive effects of CD28BP-15-ECD-Ig and CD28BP-15-ECD were comparable to or only slightly less than those of

5   CTLA-4-Ig.

Because CTLA-4-Ig has previously been shown to provide clinical benefit in patients with psoriasis, and because the immunosuppressive effects of CD28BP-15-ECD-Ig and CD28BP-15-ECD were comparable to or only slightly less than those of CTLA-4-Ig, these data support the conclusion that CD28BP-15-ECD-Ig and CD28BP-15-ECD are

10   useful as immunosuppressive agents. Importantly, because the inhibitory effects of CD28BP-15-ECD-Ig and CD28BP-15-ECD are likely to be mediated through binding to CD28, thereby preventing (blocking) B7-1 and/or B7-2 from interacting with CD28, the interaction of B7-1 and/or B7-2 with CTLA-4 in vivo during the treatment with CD28BP-15-ECD-Ig or CD28BP-15-ECD is likely to remain mostly unaffected. While CD28BP-15-

15   ECD-Ig or CD28BP-15-ECD prevent the signaling through CD28, they have little or no effect on signaling through CTLA-4. This is in contrast to CTLA-4-Ig, which binds to B7-1 and/or B7-2, preventing the interactions of endogenous B7-1/B7-2 with both CD28 and CTLA-4 in vivo. CD28BP-15-ECD-Ig and CD28BP-15-ECD were found to exhibit immunosuppressive effects and are likely to be useful in a variety of methods in which

20   immunosuppression is desirable, including, e.g., the treatment of autoimmune diseases and transplantation.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the

25   true scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. For example, all the techniques and apparatus described above may be used in various combinations. All

30   publications, patents, patent applications, and/or other documents cited in this application are incorporated herein by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated herein by reference in its entirety for all purposes.

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